

**EVALUATE AND ENHANCE SMECTITE'S EFFICIENCY IN REMOVING
AFLATOXINS FROM CORN FERMENTATION SOLUTION**

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2016

Major Subject: Soil Science

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ABSTRACT

Aflatoxins are the secondary toxic metabolites produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. They are the most studied mycotoxins because of their carcinogenicity. Corn is the major biofuel crop used to produce ethanol in the U.S. Due to stringent regulations on aflatoxins in food and feed, and increasing demand on biofuel, it appeared to be reasonable to use aflatoxin contaminated corn for biofuel production. An up to three-fold enrichment of mycotoxins in the co-product of ethanol, known as dried distiller's grain (used as animal feed), is a great concern. It would be desirable if the mycotoxins can be inactivated or removed during biofuel production.

More than two decades of studies on smectite-aflatoxin interaction proved the effectiveness of smectites as aflatoxin detoxifying agents. The ultimate goal of this study was to suppress the aflatoxin's toxicity level in animal feeds by using the environmentally safe smectites in biofuel industry. To achieve this goal, smectites were evaluated for their aflatoxin adsorption efficiencies in corn fermentation solution produced during biofuel production.

In this study, some bentonites were revealed to be effective aflatoxin binders in aqueous solution with up to ~16% adsorption capacity based on their dry mass weight. The Fourier transform infrared (FTIR), X-ray powder diffraction (XRD), and UV/Vis isotherms adsorption experiments suggested that smectites had high aflatoxin adsorption ability in ethanol and glucose solutions, two major soluble components in fermentation solution. On the contrary, the smectite's adsorption was comparatively low in real

fermentation solution. The FTIR and XRD investigations revealed the strong interlayer adsorption of organic molecules from fermentation solution in the smectites. When smectite was interacted with zein (protein extracted from corn), similar IR and XRD responses were observed. The comparative analyses suggested that proteins in fermentation solution were possibly the most interfering compounds for remarkably reduced aflatoxin adsorption. To minimize those compound's interferences on aflatoxin adsorption, smectites were modified with small nutritive organic compounds to make the interlayer space small enough to block the access of protein but large enough for aflatoxin's access. Encouragingly, the organo-smectites reduced interfering compound's adsorption but increased aflatoxin sequestration in both real and simulated fermentation solution as well as in pure protein solution.

In summary, despite strong interferences of the organic compounds, and also some minor influences from ethanol, aflatoxin adsorption by smectites in fermentation solution was still considerable, and could be even increased significantly by applying non-toxic and moreover health beneficial organo-smectites.

DEDICATION

To my beloved husband AHM MUTAHARUL ISLAM
for his support, sacrifice, and loving care

ACKNOWLEDGEMENTS

First of all, I am grateful to the Almighty Lord, who is the supreme power and authority on all over the things in the world.

I would like to express boundless thanks and deepest gratitude to my committee chair Dr. Youjun Deng for his proper guidance toward research, huge patience in reading and correcting the manuscripts, and kindness to me in my critical moment during last four years. Without his persistent support this dissertation would not have been accomplished.

My heartfelt thanks and appreciation go to the respected committee members Dr. T. D. Philips, Dr. Sam Feagley, and Dr. Joseph Awika for their effective advices and cooperation all through my degree period. My special thanks go to the department head and associate head Dr. David D. Baltensperger and Dr. Wayne Smith, respectively for their assistance whenever needed. Also thanks to my friends, colleagues, and departmental faculty and staff for making my time enjoyable and meaningful at Texas A&M University. I also want to extend my gratitude to the ‘Aflatoxin Mitigation Centre of Excellence’ for providing the financial support to conduct this research.

Finally, I am ever grateful to my husband for his inspiration, selfless support, sacrifice, and loving care which enabled me to reach my goal. My endless love and prayers to my daughters Sidratul and Samantha, and thanks for their patience to stay away from me during the last year of my study.

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CHAPTER I

INTRODUCTION

Research for aflatoxin mitigation has been accelerated with the broadcasted demonstration of the carcinogenic effects of this mycotoxin on humans and animals. Aflatoxins are the group of mycotoxins which are recognized as class A carcinogen by the International Agency for Research on Cancer (IARC, 2012). Despite of taking various preventive measures, occurrence of aflatoxin seemed to be unavoidable, especially in the drought prone regions along with some socio-economic limiting factors.

Bentonites have long been used as additives in animal feed to detoxify aflatoxin. They effectively prevented aflatoxicosis with improving the overall health and production of the poultry (Fowler et al., 2015; Kubena et al., 1998; Quisenberry, 1968). It is the smectite in bentonite, which alone plays the complete role in detoxification of aflatoxin.

Corn is extremely susceptible to aflatoxin contamination due to biotic and abiotic stresses during corn production, harvest, and storage. The Food and Drug Administration (FDA) has recommended the lowest action level of 20 ppb aflatoxin in food and feed ingredients. In the U.S., corn is the major livestock for ethanol production in the biofuel industry. Using aflatoxin contaminated corn rejected as food and feed stuff for ethanol production is seemed to be rational as no regulatory action has been taken on it. However, toxicity effects on animals might appear and eventually, humans can be affected via food chain when the livestock are being offered the aflatoxin enriched dried

distiller's grain (DDG), the co-products of ethanol. An up to three fold increment of aflatoxin in DDG compared to the aflatoxin contaminated corn used before the fermentation process is a matter of concern (Schafsma et al., 2009; Johnston et al., 2012). Consequently, though production of DDG increased dramatically with the emerging of ethanol industry, their uses are often restricted in animal industry, and acceptance in agriculture becomes limited due to the presence of mycotoxins.

To reduce the toxicity in animal feed, several physical, chemical, and biological approaches were tested. Few methods became successful to some extent in the detoxification of the contaminated corn. However, many were undesirable, and impacted negatively on fermentation process and feed safety (Sinha, 1998). To reduce the bioavailability and toxicity of aflatoxin to animals and humans, it is crucial to develop realistic, economically affordable, environmentally safe, and culturally acceptable amelioration technologies for the mycotoxin contaminated feed. If this is achieved, the adverse effects of the mycotoxins on animal, and the economic losses of the corn growers, biofuel industry, and animal industry can be minimized.

The major major goal of this study is to use the highly efficient aflatoxin binders such as smectitic clay in fermentation solution during biofuel production so that the mycotoxins would not reach the co-product in their reactive form. Incorporating clays in fermentation system and maintaining their ability to detoxify aflatoxin in fermentation solution is thought to be complex and difficult. Some practical and technical questions need to be answered: 1) Are there any compound in fermentation solution that might block aflatoxin adsorption by smectites? b) If yes, what are the interfering compounds in

fermentation solution, and how to minimize their interferences and optimize the smectite's selectivity for aflatoxin adsorption? c) what are the best ways to introduce the clays into the fermentation system? and d) how can the clays loaded with aflatoxin be separated and removed after fermentation process?.

The present study has four specific objectives as described in detail in the following chapters:

1. To investigate the physico-chemical and mineralogical properties of southern state's bentonites and fuller's earths, and to evaluate their aflatoxin B₁ adsorption efficiency in aqueous solution (Chapter III);
2. To evaluate smectite's aflatoxin B₁ adsorption efficiency in ethanol and glucose solution (Chapter IV);
3. To evaluate the smectite's aflatoxin B₁ adsorption in corn fermentation solution, and to investigate the major interfering compounds in fermentation solution that blocked aflatoxin adsorption (Chapter V); and
4. To increase the aflatoxin B₁ adsorption in real and simulated fermentation solution, and in pure protein solution by using organically modified smectites (Chapter VI).

CHAPTER II

LITERATURE REVIEW

II.1. Aflatoxins in foods and feeds

Aflatoxins are the secondary toxic metabolites produced primarily by the strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Figure 1). Aflatoxins are reported as carcinogenic chemicals to animals and humans (IARC, 2012). They were first identified after the Turkey-X disease in the 1960s that caused the death of about 100,000 turkey poultries in England due to consumption of aflatoxin contaminated peanut meal (Blount, 1961; Goldblatt, 1969).

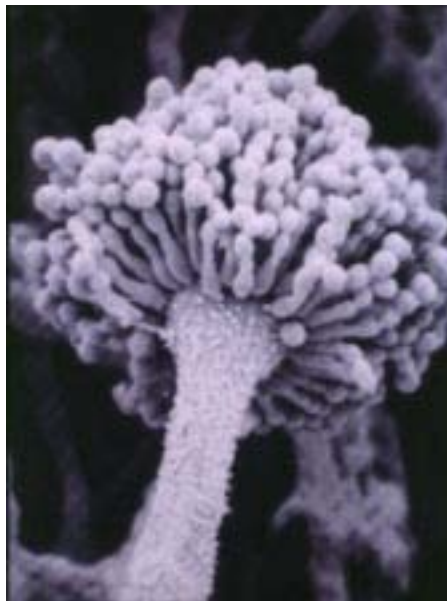


Figure 1. Electron microscopic image of *Aspergillus flavus*. Source of image: <http://poisonousplants.ansci.cornell.edu/toxicagents/aflatoxin/aflatoxin.html>.

Aspergillus flavus and *Aspergillus parasiticus* are ubiquitous in nature. These molds have been identified to produce aflatoxins in a variety of food commodities such as corn (Figure 2) and corn products, peanut and peanut products, pistachio, cotton seeds, walnut, milk, as well as in animal feeds.



Figure 2. *Aspergillus sp.* in corn from Texas. Photo by the soil mineralogy group in the Department of soil & crop sciences, TAMU.

In the southern states of U.S. corn contamination with aflatoxin seemed to be unavoidable due to frequent droughty climate. For example, corn in Texas was reported to be affected with aflatoxins at various levels during the dry period of the year (Figure 3).

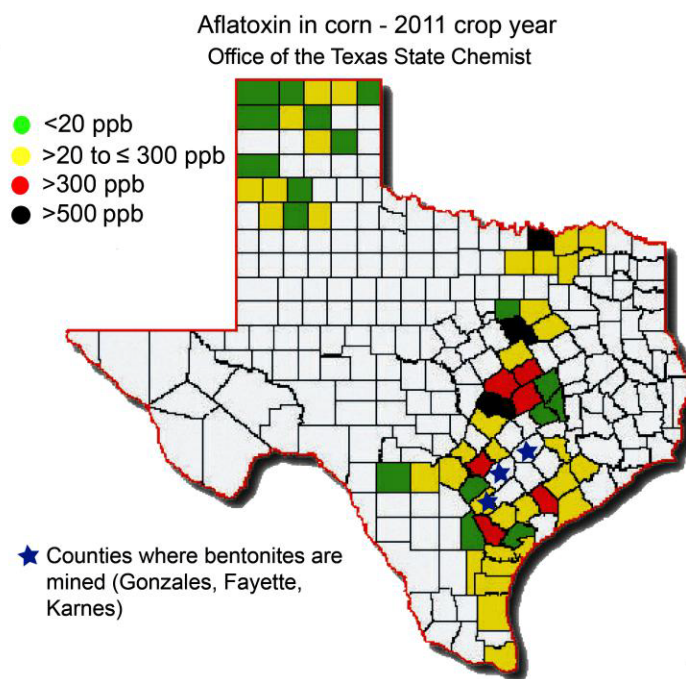


Figure 3: Aflatoxin distribution in Texas. Adopted from the aflatoxin data map of the Office of the Texas State Chemist.

Aflatoxin contamination in foods and feeds is a worldwide problem. Recently, a study on aflatoxins in food commodities in Bangladesh revealed that aflatoxin levels in five commonly ingested human food commodities such as dates, groundnuts, betel nuts, lentil, and red chili powder, and in the poultry feed were above the U.S. regulatory level of 20 ppb (Roy et al., 2013). Chestnut was found to be contaminated by the highest level ($232.9 \mu\text{g kg}^{-1}$) of aflatoxin among various foods from Istanbul (Hacıbekiroğlu and Kolak, 2013), and peanut products in Brazil also contained elevated level of aflatoxins (Magrine et al., 2011). An extremely high level of aflatoxins such as >1000 ppb in maize were recorded in Kenya in 2004 (Probst et al., 2007). The Food and Agriculture Organization (FAO) estimated that 25% of the world's food crops are affected by

mycotoxins. The US Food and Drug Administration established the action levels for aflatoxin present in human food, animal feed, and feed ingredients (Table 1, source: FDA Mycotoxin Regulatory Guidance).

Table 1. Food and Drug Administration (FDA) action levels for aflatoxin in human food, animal feed and animal feed ingredients.

| Intended use | Grain, grain byproduct, feed or other products | Aflatoxin level (ppb) |
|---|--|---------------------------|
| Human consumption | Milk | 0.5 ppb, AfM ₁ |
| Human consumption | Foods, peanuts and peanut products, brazil and pistachio nuts | 20 ppb |
| Immature animals | Corn, peanut products, and other animal feeds and ingredients, excluding cotton seed meals | 20 ppb |
| Dairy animals, animals not listed above, or unknown use | Corn, peanut products, cottonseed, and other animal feeds and ingredients | 20 ppb |
| Breeding cattle, breeding swine, and mature poultry | Corn and peanut products | 100 ppb |
| Finishing swine 100 pounds or greater in weight | Corn and peanut products | 200 ppb |
| Finishing (i.e. feedlot) beef cattle | Corn and peanut products | 300 ppb |
| Beef, cattle, swine or poultry regardless of age or breeding status | Cottonseed meal | 300 ppb |

II.2. Classification of aflatoxins

Among about 20 naturally occurring aflatoxins, aflatoxin B₁ (AfB₁) is considered as the most potential toxic and liver carcinogen (IARC, 1993; 2002). The aflatoxin B₁ is the most prevalent form among the four major groups of aflatoxins such as aflatoxin B₁, B₂, G₁, G₂ (Figure 4).

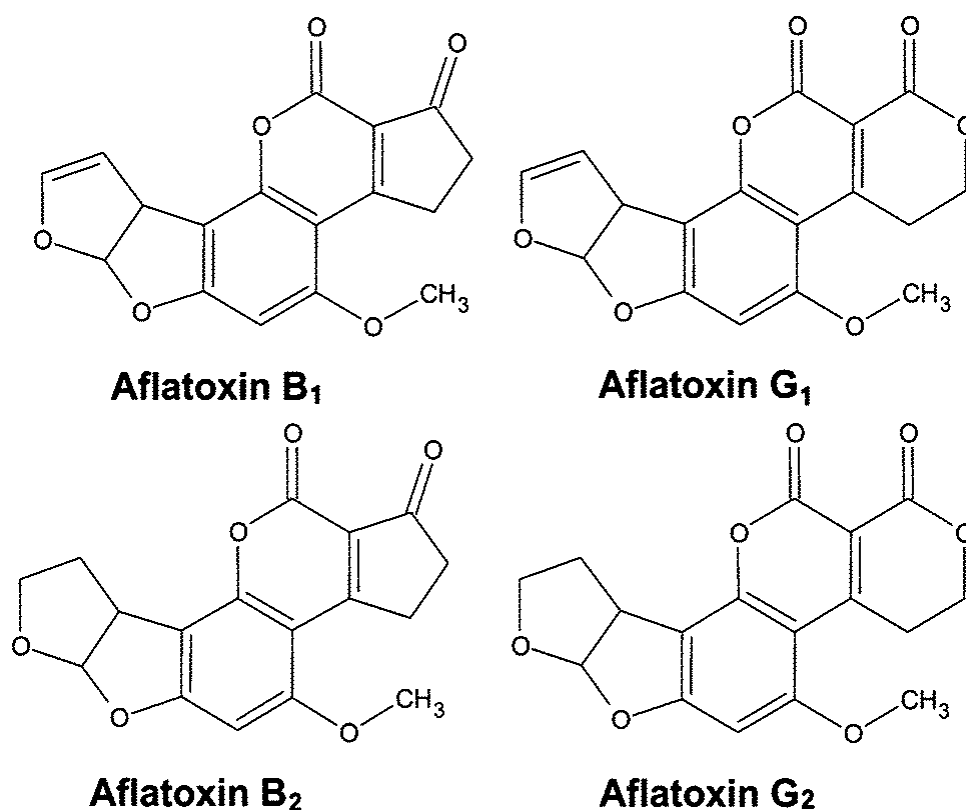


Figure 4. Molecular structures of four major types of aflatoxins. The aflatoxin B₁ and B₂ exhibit blue fluorescence, and aflatoxin G₁ and G₂ exhibit green fluorescence under UV light.

Due to carcinogenic effects on humans and animals, aflatoxin B₁ became the most intensively studied mycotoxin. The aflatoxin B₂, G₁, G₂, in addition with other two metabolic products M₁ and M₂ (contaminants in milk) were also paid much research attention due to their tendency to infect foods and feeds. The molecular weight of aflatoxin B₁ is 312.27 g mol⁻¹. It is extremely hydrophobic (solubility ~30 ppm in water).

II.3. Climates favorable for the growth of *Aspergillus species*

The fungi *Aspergillus species* grow best in hot and humid climatic conditions. The optimum temperature and moisture content for aflatoxin production depends on the

types of crops. Tentatively, temperature ranged from 24 to 35 °C, and at least 7% moisture level was found to be the ideal conditions for *Aspergillus flavus* to produce aflatoxin. In addition to climatic influences, some other biotic and abiotic factors such as improper storage, lack of irrigation facilities during drought, unavailability of pest control facilities, also accelerate the mold growth from the field to the storage.

II.4. Aflatoxin susceptible zones

Mycotoxin infections of crops and the resulting toxicity effects on animal health is recognized as a global problem. Investigations revealed that population living between 40 °N and 40 °S of the equator in developing countries are extremely susceptible to the risk of chronic and largely uncontrolled aflatoxin exposure. According to FAO, about 4.5 billion people live in this area. In addition to the climatic influences that promote mold growth and aflatoxin production, unavailability of alternative food other than the staple food such as maize in most of the African's developing countries was considered to be the major cause of aflatoxin health hazards reported in the Third Joint FAO/WHO/UNEP International Conference on Mycotoxins (Van Egmond, 1999). Moreover, lack of knowledge of the rural people about gravity of aflatoxin toxicity on human health and nutrition, insufficient facilities for preventive measures, for example, lack of technology for detection of aflatoxin in laboratories or at field, lack of expertise to detect aflatoxin, scarcity of rain and shortage of irrigation water, high cost of chemical pesticides, lack of finance to provide machineries for harvesting crops, poor facilities in drying crops for storage, etc are the limitations to control the mass exposure of aflatoxin to a vast community in a developing country.

II.5. Aflatoxicosis in humans and animals

Aflatoxicosis is a severe poisoning occurs in biological body resulting mainly from the ingestion of aflatoxin contaminated foods and feeds. The symptoms of severe aflatoxicosis are hemorrhagic necrosis of the liver, bile duct proliferation, edema, and lethargy.

The aflatoxin B₁ was responsible directly or indirectly for many health diseases and disorders both in animal and human (Coppock et al., 1989; Richard et al, 1978; Wogan, 1973). A detail information regarding the acute and chronic aflatoxin poisoning was described in a review article by Williams et al. (2004). Many studies have demonstrated a significant positive correlation between aflatoxin levels in food and liver cancer, which is known as hepatocellular carcinoma (HCC). For example, outbreaks of aflatoxicosis in Eastern and Central Provinces of Kenya took hundreds of lives (mortality rate 39%) in 2004 due to the consumption of highly aflatoxin contaminated maize (as high as >1000 ppb for some corn) which had been taken as the staple food by the rural people in Kenya since prehistoric time. Estimations of the incidence of hepatocellular carcinoma for the period 1968-74 in the Province of Inhambane, Mozambique with further investigated rate in South Africa among the mineworkers from the same province suggested that the mean aflatoxin B₁ dietary intake values for the regions studied were significantly related to HCC rates (Van Rensburg et al., 1985).

In 1974, parts of Western India experienced the outbreak of hepatitis in human due to the result of daily high consumption of corn contaminated heavily with *A. flavus* (Krishnamachari et al., 1975). The high concentration of aflatoxin in the staple diets

altered the normal nutritional pattern, suppressed growth, weakened the immunity, and eventually caused death, especially of the young generation and children (Denning et al., 1995; Gong et al., 2003; Oyelami et al., 1997). Adults had more tolerance level of acute aflatoxin poisoning than children (Cullen and Newberne, 1993).

In animals, aflatoxins were also detected to be carcinogenic, mutagenic, teratogenic, and immune suppression. Among all livestock, the poultry was reported to be most adversely affected by the aflatoxin poisoning in various ways. Some examples of the health hazards were reduced weight gain, decreased egg production, poor pigmentation, and liver damage (Devegowda and Murthy, 2005; Leeson, et al., 1995). Aflatoxin exposure to poultry through their diet showed to decrease the antibody response of the vaccine (Azzam and Gabal, 1998). The vitamin A and D level in the broiler chickens were also found to be affected by the aflatoxin ingestion with feed (Glahn et al., 1991; Reddy et al., 1989). Rustemeyer et al. (2010) in their study concluded that the performance and health of young growing barrows were affected by consumption of aflatoxin B₁ contaminated diet, especially when fed for a more extended period.

II.6. Use of mycotoxin contaminated corn in biofuel industry, three-fold mycotoxin enriched DDG, and economical loss

More than one third of the corn produced in the US is used as feedstock for ethanol production (USDA, 2015). According to independent statistics and analysis of US Energy Information Administration (EIA, 2015), the country produced 14.34 billion gallons of ethanol in 2014. Corn contributed more than 80% of the entire ethanol

production. Due to high market price of corn, mycotoxin contamination, and rapid growth of biofuel industry, it seemed to be rational to use the aflatoxin contaminated corn for biofuel production. Such use might be suggested to reduce wastage of corn from the economical perspective.

The co-products of ethanol production or DDG (Figure 5) are extensively used worldwide due to their large availability, low cost, and high nutritive value. The DDG is high in energy, contain digestible phosphorus, and contributes a significant source of proteins to the livestock. The mycotoxin contamination often threatened their suitability to be used as animal feeds. Three times higher concentration of mycotoxins than the original contaminated corn were detected in DDGs during biofuel production (Bennett and Richard, 1996; Bothast et al., 1992; Schaafsma et al., 2009; Wu and Munkvold, 2008).



Figure 5. White Energy Inc. biofuel industry in Plainview, Texas. Aflatoxin will be enriched in DDG if contaminated corn is used for ethanol production. Photo was taken by the soil mineralogy group in the department of soil & crop sciences, TAMU.

In biofuel industry, one third of the corn mass is consumed for ethanol production, one third produces carbon dioxides, and the rest one third produces the co-product. Recent studies on the fate of aflatoxin in corn fermentation confirmed that aflatoxin does not enter the distilled ethanol during biofuel production. On the contrary, most of the toxins were found in the dried corn solids at the end of the fermentation process (Johnston et al., 2012).

The ethanol and animal industries situated in close proximity to aflatoxin-contamination susceptible regions might have greater concern. However, not all the US DDG were detected to have mycotoxin level beyond the FDA regulatory limit (Zhang et al., 2009).

Other mycotoxins (fumonisin, zearalenone, ochratoxin, etc) can occur in DDG too. It was estimated that current loss in a swine industry for the weight reduction due to having fumonisins in fed DDG was in an average of \$9 million annually (Wu and Munkvold, 2008). The author concluded that for the total mycotoxins, if not controlled, the economical loss would be significantly higher than the single mycotoxin due to multiple adverse health effects on animals. Therefore, it is necessary to develop some realistic and beneficial strategies to minimize the mycotoxin concentration in DDG during biofuel production so that the adverse toxicity effects on animals and in turn, on humans can be reduced, and economic loss of the animal growers can be minimized. The safe and highly effective aflatoxin binders such as bentonite clays could play a very significant role in minimizing the aflatoxin poisoning to livestock through reducing the toxicity in animal feeds during biofuel production.

II.7. Clay minerals

“A mineral is a naturally occurring homogeneous solid with a definite chemical composition and a highly ordered atomic arrangement, which is usually formed by inorganic processes” (Klein and Hurlbut Jr, 1993a). Minerals possess many functions which have physical, chemical, and biological importance for soil-plant system. Soil minerals are the store house of nutrients for plant, and microbes.

Mineralogists have classified minerals into several classes based on their chemical compositions. The classes are native elements, sulfides, sulfosalts, oxides and hydroxides, halides, carbonates, nitrates, borates, phosphates, sulfates, tungstates, and silicates (Klein and Hurlbut Jr, 1993b). Soil minerals are also divided into two broad

categories such as primary and secondary minerals. Primary minerals are formed at elevated temperature and inherited from igneous and metamorphic rocks. Secondary minerals are formed by low temperature and derived from either sedimentary rocks or formed in soil by weathering (Jackson, 1964).

The silicate minerals are recognized as the broadest and most important group of minerals. They constitute more than 90% of the earth's crust, and about 40% of the minerals in soil are silicates (Schulze, 2002). Within this group, phyllosilicates are known to play the vital key role in soil fertility and plant nutrition due to their small particle size, large surface area, and distinct cation exchange properties. They are generally called clay minerals. The basic building blocks in the structure of phyllosilicate minerals are the tetrahedron and octahedron (Figure 6).

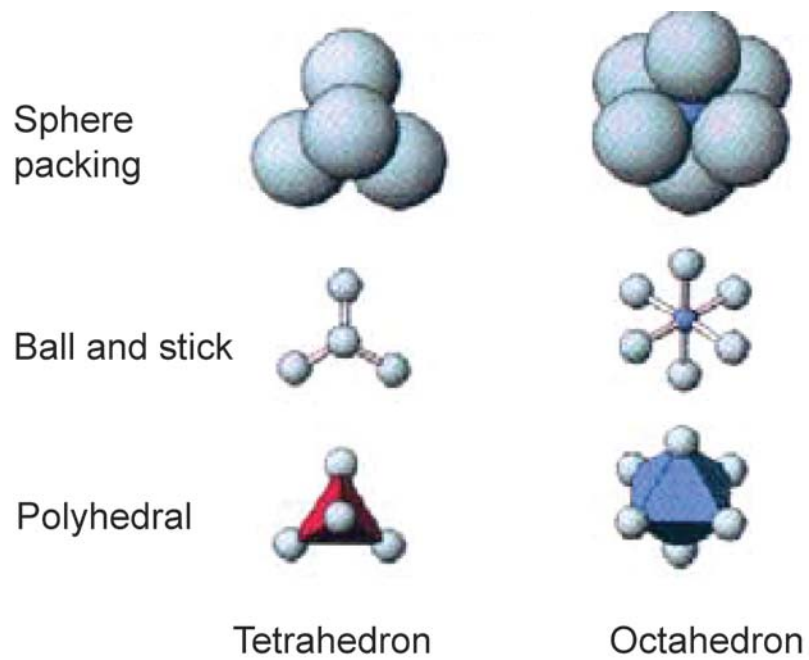


Figure 6. The primary building blocks in common soil minerals presented in three different ways (Adopted from Plate 1-3 in Chapter 1, D. G. Schulze, 2002).

II. 8. Smectite: a versatile phyllosilicate clay mineral

Smectite is a group of expansive 2:1 layer phyllosilicate minerals, which is the dominant mineral in bentonite's structural composition (Grim, 1962). Smectites have the layer charge varies from 0.20 to 0.60 per formula unit. The 2:1 layer is consisted of one octahedral sheet sandwiched between two tetrahedral sheets. The tetrahedral sheet contains SiO_4 tetrahedra of which three O^{-2} ions of each tetrahedron share with three neighbor tetraherda (Figure 7).

Smectite is classified according to octahedral cations, location of isomorphic substitution, and whether the mineral is di or trioctahedral. The arrangement of octahedral sheets is presented in the Figure 8. Dioctahedral smectites are the most common in soils. The montmorillonite, beidellite, and nontronite are the three major minerals that represent the dioctahedral smectite. The saponite, sauconite, and hectorite are the trioctahedral smectites, which are less common in soils. The substitution of one cation for another in the octahedral and tetrahedral sheet produces the layer charge. In montmorillonite, substitution of Mg^{+2} for Al^{+3} occurs in the octahedral sheets. In beidellite and nontronite, substitution of Al^{+3} for Si^{+4} occurs in the tetrahedral sheet. The structural unit of phyllosillcate mineral is presented in the Figure 9.

Smectites are versatile clay minerals in nature. They can hold water and exchangeable cations in their interlayer and can expand to even more than 1.8 nm (Figure 10). They can adsorb many compounds either organic or inorganic including contaminants from the environments.

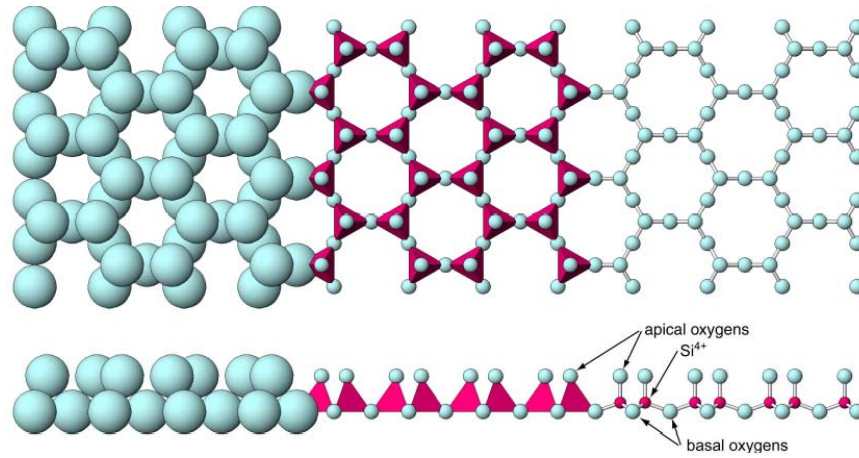


Figure 7. The ideal tetrahedral sheet arrangement in phyllosilicate minerals. Adopted from Plate 1-5 in Chapter 1, D. G. Schulze, 2002).

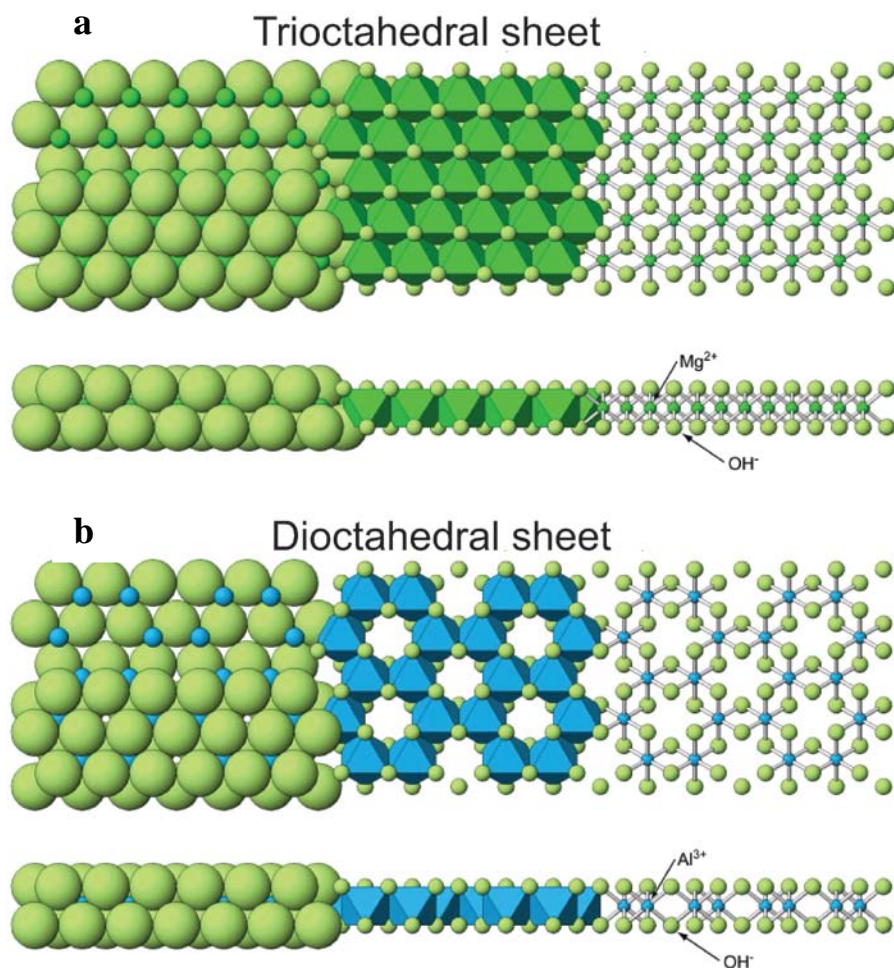


Figure 8. The octahedral sheet. (a) A trioctahedral sheet. (b) A dioctahedral sheet.
Adopted from Plate 1-4 in Chapter 1, D. G. Schulze, 2002.

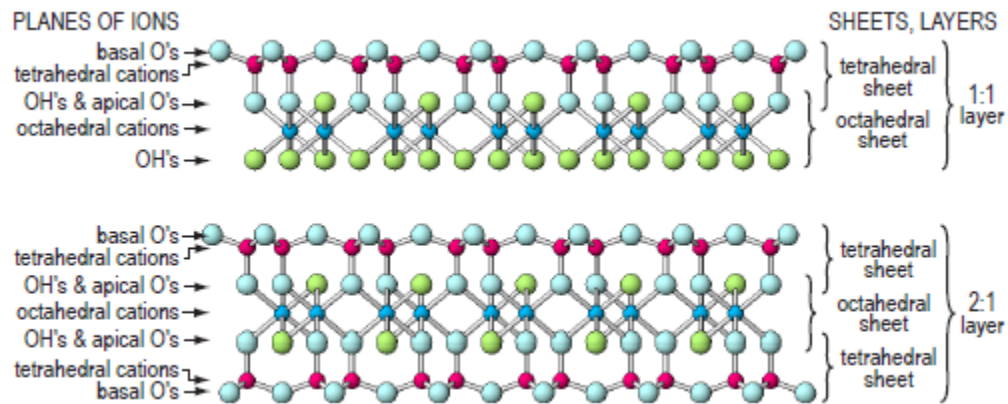


Figure 9. Edge view of the 1:1 and 2:1 layer structures illustrating phyllosilicate nomenclature. Adopted from Plate 1-6 (b) in Chapter 1, D. G. Schulze, 2002).

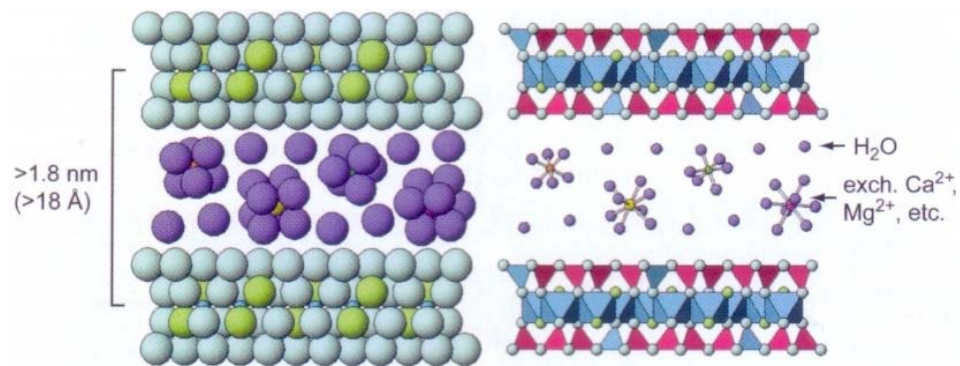


Figure 10. The 2:1 layer structural scheme of smectite. The cations and water molecules are in the interlayer. Adopted from Plate 1-8 in Chapter 1, D. G. Schulze, 2002).

II.9. Smectites: the detoxifying agents for aflatoxins

Smectites are the safe and highly effective adsorbents for aflatoxins, and have been used successfully in animal and human clinical trials for more than two decades (Desjardins et al., 2003; Diaz et al., 2004; Dixon et al., 2008; Fowler et al., 2015; Jaynes and Zartman, 2011; Magnoli et al., 2008; Marroquín-Cardona et al., 2009; Phillips, 1999; Phillips et al., 2008; Wang et al., 2008).

The commercial product, Novasil clay, which is a processed bentonite, was evaluated as aflatoxin detoxifying agents both *in vitro* and *in vivo* systems in many studies (Phillips, 1999; Phillips et al., 2008, 1987). *In vitro* evaluations helped to screen the potential binder products based on their binding affinity and capacity. *In vivo* studies assisted to investigate the performance responses or biological markers such as tissue residues or changes in biochemical parameters to determine effectiveness of binders.

Consumption of aflatoxin contaminated diet treated with smectites even at low concentration of 0.5% showed to reduce the bioavailability of aflatoxin in farm animals including chickens, turkey poults, pigs, cows, goats, lambs, and minks (Ramos and Hernández, 1997). The smectite at 0.25 or 0.375% could also protected the young broiler chickens from aflatoxicosis (Kubena et al., 1998). Recent investigation on the effects of calcium bentonite clay in aflatoxin contaminated diet on performance of starter broiler chicks suggested that 0.2% clay was effective in reducing the accumulation of aflatoxin B₁ residues in the liver and improving livability in birds fed aflatoxin (Fowler et al., 2015). A sodium bentonite at 1.2% level mixed with aflatoxin B₁ contaminated feed significantly reduced the toxicity of aflatoxin M₁ in cow's milk due to less transfer of

aflatoxin B₁ to its metabolite (Diaz et al., 2004). Research showed when the smectite was added to the diet of leghorn and broiler chicks at a level of 0.5%, significantly reduced the adverse effects of feeding 7.5 mg AfB₁ kg⁻¹ of feed, and suggested that the aluminosilicates were effective preventive management of aflatoxicosis (Phillips et al., 1987). Smectites were found to decrease the aflatoxin B₁ toxicity in rumen fermentation of a hay-rich feed mixture (Jiang et al., 2014).

Research demonstrated the relative safety of the NovaSil clay in human body if consumed with diet for a certain period, and suggested to serve this clay as a basis for a long-term human trials in populations at high risk for aflatoxicosis (Wang et al., 2005). Continued investigations on Ghanaians proved that NovaSil clay significantly reduced the concentrations of AfB₁-albumin adduct in blood samples and aflatoxin M₁, a metabolite of aflatoxin B₁ in urine samples (Wang et al., 2008). Phillips et al. (2008) in a review article broadly described the positive and significant effects of the Novasil clay to reduce aflatoxicosis in humans when the clay was taken as dietary supplement. The authors recommended the clays for their safety and effectiveness in human body due to showing no adverse effects on health, and negligible interactions with serum vitamin A and E, iron, zinc and other micronutrients.

To confirm the safety of clay in human body, a study was conducted using montmorillonite in animal and suggested that montmorillonite could cause some cytotoxic effects at high concentration after long-time exposure but no remarkable toxicity was found in mice treated with high dose up to 1,000 mg kg⁻¹. The researchers

expressed their consent to the sustainable contribution of montmorillonite clay as an oral delivery carrier with safe level (Baek et al., 2012).

II.10. Bonding mechanism between aflatoxin B₁ and smectite

Researchers proposed different bonding mechanisms for aflatoxin adsorption by smectite. There is still argument remaining on what mechanism plays the dominant role for aflatoxin adsorption. It is well documented that aflatoxin's adsorption occurred mainly in the interlayer region of smectites (Deng et al., 2010; Grant and Phillips, 1998), although it was claimed that aflatoxin B₁ was chemically adsorbed at external basal surface and edges by the smectite (Grant and Phillips, 1998). Aflatoxin adsorption capacity of the smectites decreased from 0.336 to 0.0567 mol kg⁻¹ when the smectite was collapsed by heating, suggested the primary adsorption of aflatoxin B₁ was in the interlayer of clay (Phillips, 1999). Again, the basal spacing of aflatoxin B₁ saturated smectites exhibited greater resistance to collapse on heating than untreated smectites indicating that aflatoxin B₁ entered the interlayer galleries of the smectites (Kannevischer et al., 2006). Deng et al. (2010) proposed that aflatoxins occupied the interlayer of smectite with exchange cations and water molecules (Figure 11).

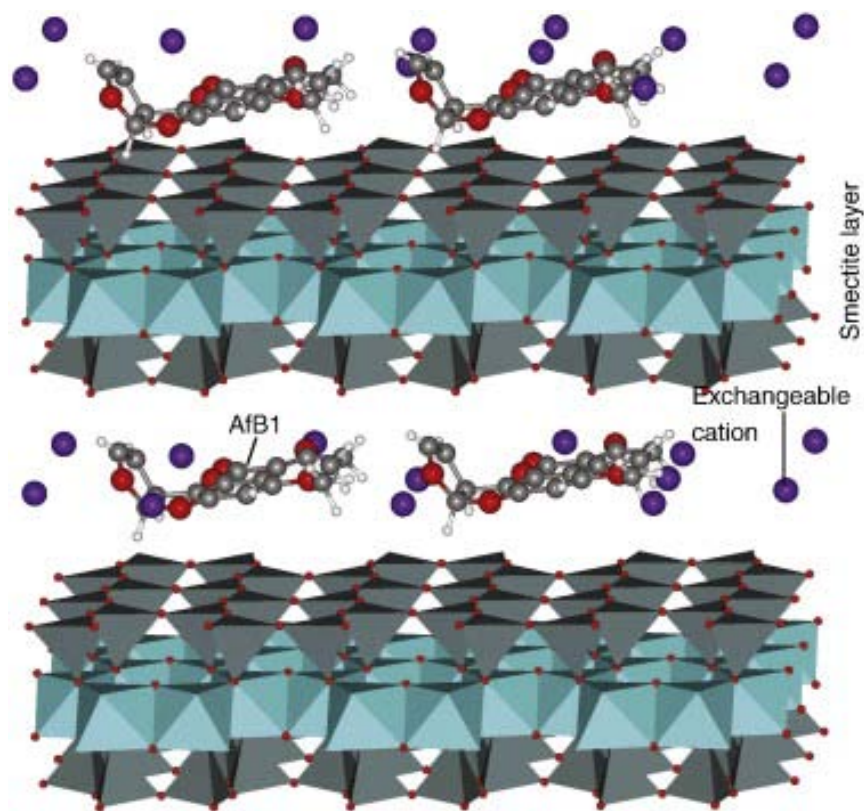


Figure 11. Proposed model of an aflatoxin intercalated smectite. Interlayer water molecules were not shown. Adopted from Deng et al. (2010).

The several proposed bonding mechanisms between aflatoxin B₁ and smectite were described in detail by Deng et al. (2010). Some models are briefly described below:

II.10.1. Selective chemisorptions

According to Phillips et al. (1995), carbonyl stretching bands of aflatoxin between 1700 and 1750 cm⁻¹ disappeared when the toxin reacted with the smectite, and new bands appeared between 1400 and 1600 cm⁻¹. They suggested that the carbonyl groups in aflatoxin formed mononuclear bidentate chelates with the transition metals in clay.

II.10.2. Electron donor-acceptor (EDA) model

The concept of this model was adopted from Haderlein et al. (1996), and was suggested to be the most possible leading mechanism for aflatoxin B₁ adsorption by smectite (Phillips, 1999; Phillips et al., 2008). They explained that the two carbons of two carbonyl groups in aflatoxin molecule possess partial positive charges that could be bonded with the negatively charged smectite.

II.10.3. Direct ion-dipole interaction and hydrogen bonding model

Perhaps, the model proposed by Deng et al. (2010) is the most recent one that discussed the bonding mechanisms between aflatoxin B₁ and smectite with the evidences of IR spectroscopy. They suggested two bonding mechanisms were underlying aflatoxin adsorptions: 1) under dry condition, major bonding between adsorbed aflatoxin B₁ and smectite was direct ion-dipole interactions or coordination between exchange cation and

two oxygen of carbonyl groups, and 2) under humid condition, H-bonding between carbonyl groups and hydration-shell water surrounding exchange cation (Figure 12).

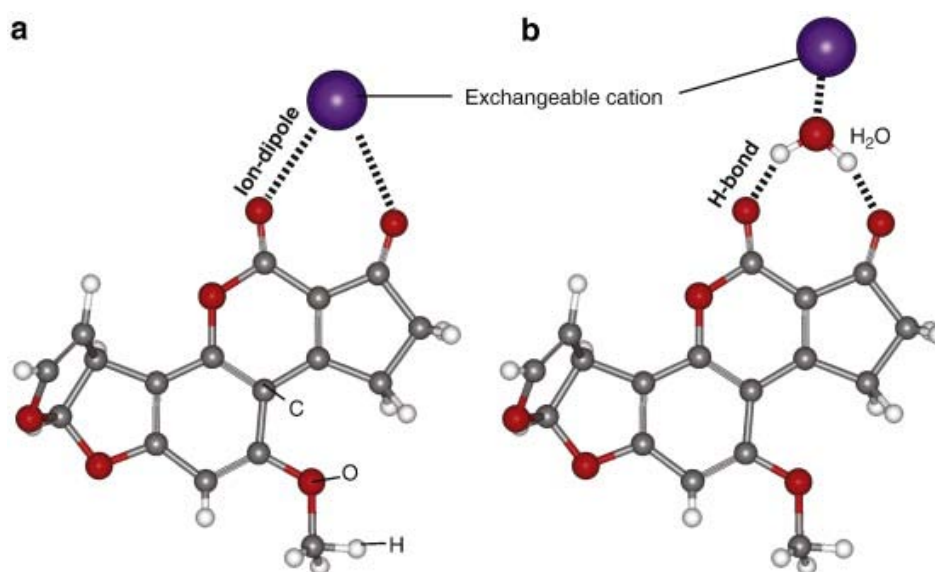


Figure 12. Proposed bonding mechanisms: (a) ion-dipole interaction/coordination between the two carbonyl oxygens with exchange cation, and (b) H-bonding between carbonyl functional groups and exchange-cation hydration-shell water. Adopted from Deng et al. (2010).

Deng et al. (2010) showed that at ~100% humidity, types of cations had no effects on aflatoxin-smectite infrared bands positions. On the contrary, at ~0% humidity, cations had remarkable effects on aflatoxin B₁ band's shifting on smectite. They revealed that in the ion-dipole interaction mode, the cations with higher valence, smaller size or with unpaired electrons in d-orbits showed stronger bonding with aflatoxin than the other cations.

Computational evaluation of bonding between aflatoxin B₁ and smectite confirmed the importance of carbonyl groups in the bonding of aflatoxin to smectite and revealed more subtle interactions between exchange cations and the dihydrofuran oxygen (Deng and Szczerba, 2011). Study revealed that selecting a smectite with adequate charge density or by replacing the exchange cations with divalent cations that have lower hydration energy enhanced the selectivity of smectites for aflatoxin (Deng et al., 2012). FTIR investigations indicated that the octahedral Fe in smectite and amorphous silica in the clays both were responsible for greater aflatoxin B₁ adsorption potential (Tenorio et al., 2008).

CHAPTER III

MINERALOGY AND AFLATOXIN B₁ ADSORPTION EFFICIENCY OF SOME U.S. BENTONITES AND FULLER'S EARTH

III.1. Introduction

Bentonite is a soft clayey rock which was first identified in cretaceous rocks in Wyoming, and was derived from the deposit of weathered volcanic ash (Senkayi et al., 1985). Bentonite contains the aluminum phyllosilicate minerals, of which mostly are the montmorillonite. The most popular definition of bentonite was given by Ross and Shannon (1926) as follows:

“Bentonite is a rock composed essentially of a crystalline clay-like mineral formed by devitrification and the accompanying chemical alteration of a glassy igneous material, usually a tuff or volcanic ash: and it often contains variable proportions of accessory crystal grains that were originally phenocrysts in the volcanic glass. These are feldspar, biotite, quartz, pyroxenes, zircon, and various other minerals typical of volcanic rocks. The characteristic clay-like mineral has a micaceous habit and facile cleavage, high birefringence and a texture inherited from volcanic tuff or ash, and it is usually the mineral montmorillonite, but less often beidellite”. Devitrification is the process of crystallization in a formerly crystal-free (amorphous) glass. The term is derived from the Latin vitreus, meaning glassy and transparent.

Recently U.S. Geological Survey, Mineral Commodity Summaries reported that U.S. produced 4.8×10^{11} kilogram of bentonites in 2012, representing ~48% of the

world's total bentonite production. Alabama and Mississippi bentonite together produced at least one fifth of the total production in US. Most bentonites mined in Texas, Alabama and Mississippi were calcium bentonites.

Fuller's earth is the clay material consisting primarily of the mineral attapulgite or palygorskite. They are the typical clay also available in the southeastern US. The palygorskite is the magnesium aluminium phyllosilicate mineral, often contains smectites to some extent. The ideal formula of palygorskite is $\text{Si}_8\text{Mg}_5\text{O}_{20}(\text{OH})_2(\text{OH}_2)_4 \cdot 4\text{H}_2\text{O}$. It has the same basic 2:1 layer structure as smectite but their apical oxygen in the tetrahedral sheet invert periodically such that a modulated layers structure formed as ribbons of 2:1 layers joined at their edges (Figure 13). Tunnels formed in this modulated structure have a width of ~ 0.89 nm and a height of ~ 0.68 nm. The tunnels appear to be large enough to host aflatoxin molecules. Palygorkites adsorbed different cationic dyes from aqueous solutions (Al-Futaisi et al., 2007), and was reported to adsorb non-polar organic compounds (Singer, 2002).

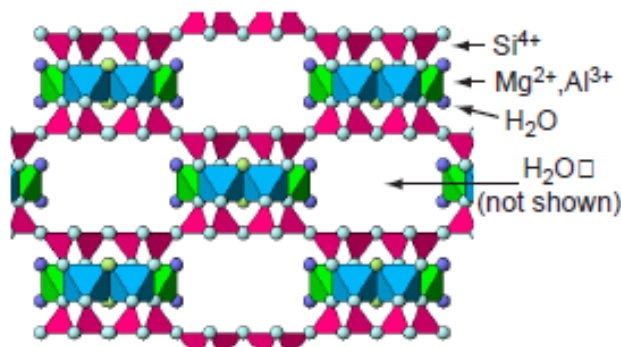


Figure 13. Structural model of palygorskite. Adopted from Schulze (2002).

In the present study some general physico-chemical and mineralogical properties were investigated for some bentonites and palygorskites. Aflatoxin adsorption capacities were evaluated for the extracted smectites from the clays. In the U.S., the local bentonite sources might be an ideal or practical solution for the detoxification of aflatoxin contaminated food and feed. Therefore, the major goal of the present study was to evaluate the aflatoxin adsorption efficiency of some calcium bentonites and palygorskites with their basic mineralogical characteristics.

The specific objectives were: 1) to investigate the minerals in the bentonite samples by X-ray diffraction (XRD), 2) to investigate the bentonites and smectites by the Fourier Transmission Infrared (FTIR), 3) to study the samples by SEM and TEM technology, and 4) to evaluate the aflatoxin B₁ adsorption ability of the smectites in aqueous solution by the UV/Vis-spectrophotometer.

III.2. Materials and Methods

III.2.1. Sources of bentonites and palygorskites

Four bentonites and two fuller's earth samples were investigated in this study. Bentonite 2MS, 3MS, and 4MS were originated in Mississippi. They were commercially branded as NovaSilTM, and provided by the Office of Texas State Chemist (OTSC). Bentonite MBBO1 was mined in Alabama and supplied by American Colloid Company. Fuller' earths Acti-Gel 208 and MUG-FG was collected from the Antioch Church Road mining area near the town Attapulugus, Georgia. They were offered by Active Mine. The Acti-Gel 208 was a purified version of the MUG-FG.

III.2.2. Measurement of pH and EC

The pH and EC of the bulk samples were recorded. The Thermo Scientific Orion 370 PerpHecT® LogR® pH/ISE was used for pH, and YSI 3100 conductivity meter was used for EC measurement. One gram of bentonite from each of the six samples was suspended into 20 mL deionized water contained in 50 mL centrifuge tube. The suspensions were shaken for 1 hour on a rotary shaker, and centrifuged at 2000 rpm for 10 minutes. The clear supernatant was measured for EC followed by pH to avoid addition of any electrolytes (e.g., KCl) that can release from pH electrode.

III.2.3. Pretreatment and size fractionation

All the raw bentonites and palygorskites were size fractionated to separate out the sand, silt, and clay particles. Five gram of samples was taken for the treatment. Before size fractionation, the cementing materials such as calcite and dolomite in the samples were removed by treating with 1M pH 5 NaOAc buffer solution. The samples did not show reaction with hydrogen peroxide, indicating the almost absence of organic matter, manganese oxides, or sulfides.

The treated samples were then fractionated into various size classes by repeatedly washing with diluted pH 10 Na_2CO_3 solution. The sand particles ($>53\mu\text{m}$) were separated first and collected by sieving. Then the silt ($2\text{-}53\mu\text{m}$) particles were collected. Finally, the clay fractions ($<2\mu\text{m}$) were dialyzed and collected by centrifugation based on the Stokes's law (Deng et al., 2012). The sand and silt fractions were oven dried at $105\text{ }^\circ\text{C}$ overnight. The sodium saturated stock clay suspensions were preserved in the

refrigerator for further use. A portion of clay suspension was treated with 1N CaCl_2 to replace the Na by Ca ion for the aflatoxin adsorption experiment.

III.2.4. Determination of cation exchange capacity (CEC)

CEC of the clays were determined by displacement of adsorbed exchangeable ions with 0.5 M CaCl_2 , and by reaction with 0.005 M CaCl_2 to dilute the interstitial Ca^{2+} solution. This prevented water hydrolysis and exchange of H^+ for Ca^{2+} on adsorbed colloidal exchange sites. Calcium ions were subsequently displaced with Mg^{2+} by treating with 0.5 M MgCl_2 solution. The quantity of displaced calcium ions in solution was then measured with atomic absorption spectroscopy (AAS) in duplicate, and after correcting for calcium interstitial solution, used to calculate CEC (Deng et al., 2012).

III.2.5. X-ray diffraction (XRD) procedures

All the mineralogical analyses (XRD, FTIR, SEM, & TEM) were performed according to Deng et al. (2012).

The bulk, sand, silt, and clay fractions of the samples were analyzed by the XRD. For sand analysis, 1 gram of a representative ground sand was passed through a <140 mesh sieve. For silt analysis, no grinding was required as the silt was smaller than the 140 mesh sieve. The samples were placed on an XRD mount cavity. They were analyzed by the Bruker D8 Advance X-ray diffractometer from 2 to 70° 2 θ using CuK_α radiation. Clay fractions were saturated with Mg and solvated with glycerol before placing on XRD. This saturation provided a known cation which stabilizes the d_{001} spacing for vermiculites and smectites. This allowed identification of phyllosilicates. Part of the clay

suspension was transferred by a disposable pipette on the surface of a glass disc and allowed to air-dry before loading on sample holder for XRD analyses.

III.2.6. Fourier transmission infrared (FTIR) procedures

The bulk samples were analyzed by diffuse reflectance infrared transform (DRIFT) accessory. The clays were analyzed as pressed pellet by transmission method. For bulk IR analyses the samples were prepared by mixing ~0.01 gram of sample with 0.3 gram of KBr in a Wig-L-Bug® (Lyons, III 60534) mixture for 30 seconds. The KBr was the background material. Same procedure was followed for mixing clay (0.001gram) with KBr. The mixture was transferred to the pellet die chamber and pellet was formed under high pressure. The FTIR spectra were recorded under the Perkin Elmer Spectrum 100 infrared spectrometer.

III.2.7. Scanning electron microscope (SEM) and transmission electron microscope (TEM)

An FEI QUANTA 600F field emission scanning electron microscope was used to exam the silt (2-53 μ m) particles. The particles were coated with 4 nm thick of Pt/Pd (platinum/palladium) alloy by sputter coating. An FEI Tecnai™ G2 ST field emission transmission electron microscope operated at an acceleration voltage of 200 KeV was used to exam the clay particles mounted on holey carbon TEM grids.

III.2.8. Procedure for UV measurement of aflatoxin B₁ adsorption

The sample preparation and analyses for the isothermal adsorption of aflatoxin B₁ by smectites was performed by following the procedures described by Kannewischer

et al. (2006). The powder form of aflatoxin B₁ (50 mg) from *Aspergillus flavus* was purchased from Sigma Chemical Co. (St. Louis, MO 63118, USA), CAS No. 1162-65-8. A high purity (>99%) acetonitrile (LC-MSchromasolv®) of HPLC grade was purchased from Sigma Aldrich, CAS No. 75-05-8.

A 1000 ppm stock solution was prepared by dissolving 50 mg of aflatoxin B₁ in 50 mL acetonitrile in a glass volumetric flask. The mouth of flask was covered by parafilm to protect the solution from evaporation. The flask was wrapped with aluminum foil before storing it in the refrigerator at 4°C. The 8 ppm aflatoxin B₁ working solution was prepared from stock solution. For batch adsorption experiments, 0.0, 0.4, 1.6, 3.2, 4.8, 6.4, and 8.0 ppm concentrations were prepared for both the standard curve and samples with 2 replications. To each 15 mL Nalgene Falcon™ centrifuge tube containing 5 mL of aflatoxin B₁ solution, 50 µL of calcium smectite suspension containing 0.1 mg clay was added. Same procedure was followed for the blank without adding clay. The mixtures were shaken for 24 hours on a rotary shaker at 200 rpm. The tubes were centrifuged at 4500 rpm for 57 minutes. The absorbance of aflatoxin B₁ in clear supernatant was measured with the Beckman Coulter DU800 UV/visible-spectrophotometer at 365 nm wavelength (Kannevischer et al., 2006).

III.3. Results and discussions

III.3.1. Preliminary screening of samples studied

Some general physical and chemical properties of the samples were analyzed at their original condition (Table 2). For some samples although on an average, 15% of the

materials were lost during size fractionation, the samples still had at least ~65% of clay particles based on weight. The Acti-Gel 208 was almost clayey (~95%).

The bentonites had pH ranged from 8.0 to 8.5 indicating calcareous rich substances were present in those samples. The palygorskites had pH 9.9 suggesting these clay minerals were highly saturated by sodium ions. The EC of Acti-Gel 208 was remarkably higher than other samples. The CEC was recorded at between 85.4 and 94.3 cmol kg^{-1} for the bentonites. However, the CEC of palygorskites were not determined due to some limitations.

Table 2. Some general properties of bentonites and palygorskites.

| Sample | Size fractionation (%) | | | pH | EC ($\mu\text{S cm}^{-1}$) | CEC NaOAC (cmol kg^{-1}) |
|--------------|-------------------------------|--------------------------------|------------------------------|-----|---------------------------------|---|
| | Sand ($>53 \mu\text{m}$) | Silt ($2-53 \mu\text{m}$) | Clay ($<2 \mu\text{m}$) | | | |
| 2 MS | 6.04 | 9.07 | 65.42 | 8.4 | 180 | 85.4 |
| 3 MS | 6.71 | 22.02 | 69.87 | 8.3 | 148 | 94.3 |
| 4 MS | 6.78 | 10.37 | 65.30 | 8.5 | 159 | 93.3 |
| MBBO1 | 9.95 | 14.49 | 64.03 | 8.0 | 168 | 89.5 |
| Acti-Gel 208 | 0.01 | 3.14 | 95.0 | 9.9 | 307 | - |
| MUG-FG | 5.78 | 12.23 | 75.54 | 9.9 | 91 | - |

III.3.2. XRD revealed the presence of smectite in all bentonites

XRD results indicated that the bentonites were saturated with smectites. The most distinctive or sharp peak of smectite at d_{001} spacing was recorded at ~ 1.5 nm in all bentonites. The other minor peaks of smectite were found at 0.45, 0.25, and 0.15 nm. The raw bentonites also contained other minerals e.g., quartz, pyrite, muscovite, and calcite (Figure 14).

The quartz (SiO_2) was identified by its distinctive reflections at 0.33 nm, followed by 0.426, and 0.182 nm. The pyrite (FeS_2) peaks were investigated at 0.27, 0.19, and 0.16 nm. A very low intensified peak 0.99 nm suggested the presence of muscovite. Calcite (CaCO_3) was identified by only a weak reflection at 0.302 nm. A very low intense d_{001} peak of smectite (~ 1.5 nm) was found in the spectra of MUG-FG but Acti Gel 208 was completely devoid of this peak. These samples were dominated by their primary mineral palygorskite $[(\text{Mg},\text{Al})_2\text{Si}_4\text{O}_{10}(\text{OH})\cdot 4(\text{H}_2\text{O})]$ and showed peak at 1.05, 0.64, 0.54, and 0.44 Å.

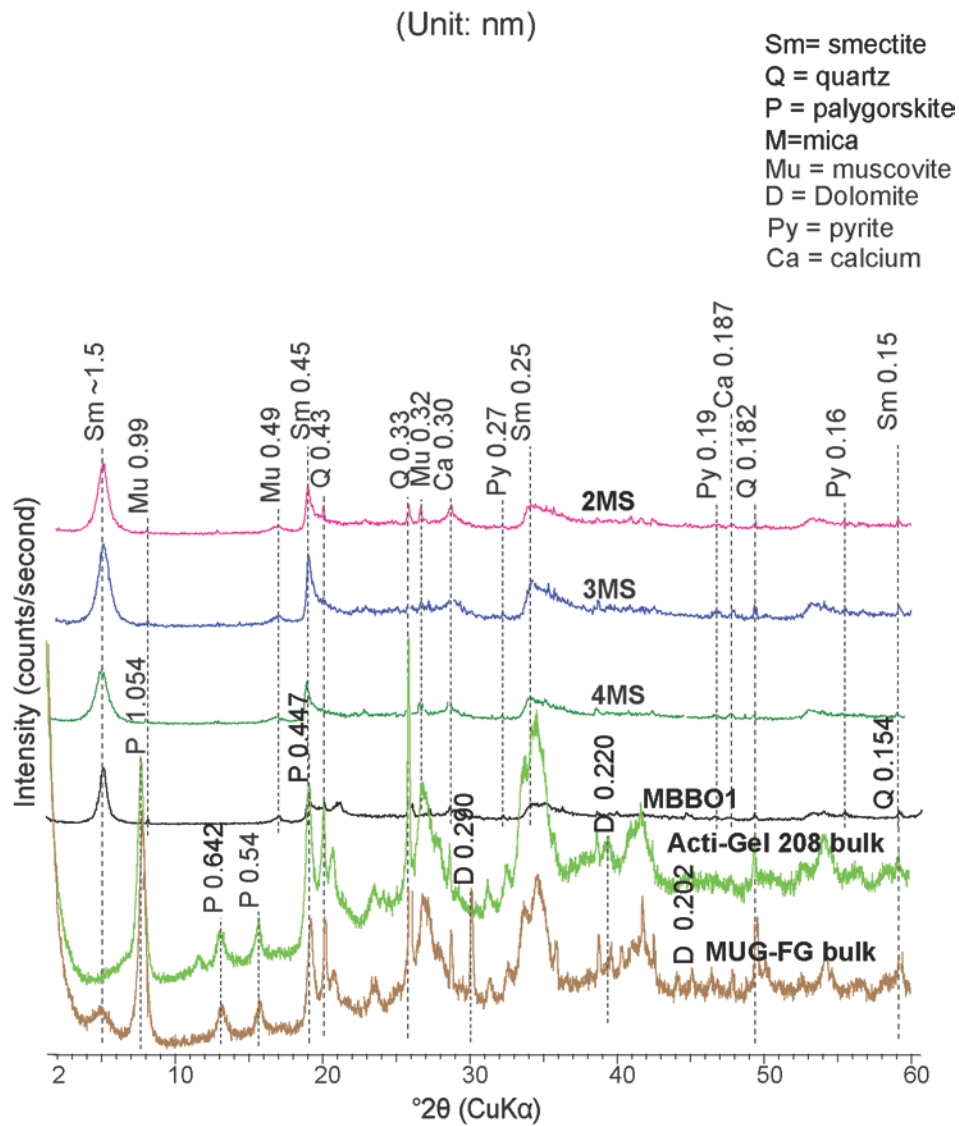


Figure 14. Minerals identified in the bulk bentonites and palygorskites by XRD.

The peak of smectite may vary from 1.2 to 1.5 nm at natural condition. Smectites were present abundantly in the clay fractions of bentonites, revealed by their expansion up to 1.80 nm when the Mg saturated clay was treated by glycerol salvation (Figure 15). Palygorskites contained negligible fraction of smectites as reflected by low intensified peak at d_{001} position. They were dominated by palygorskites (first peak at 1.06 nm) with minor kaolinite (0.72 nm) and quartz (0.33 nm).

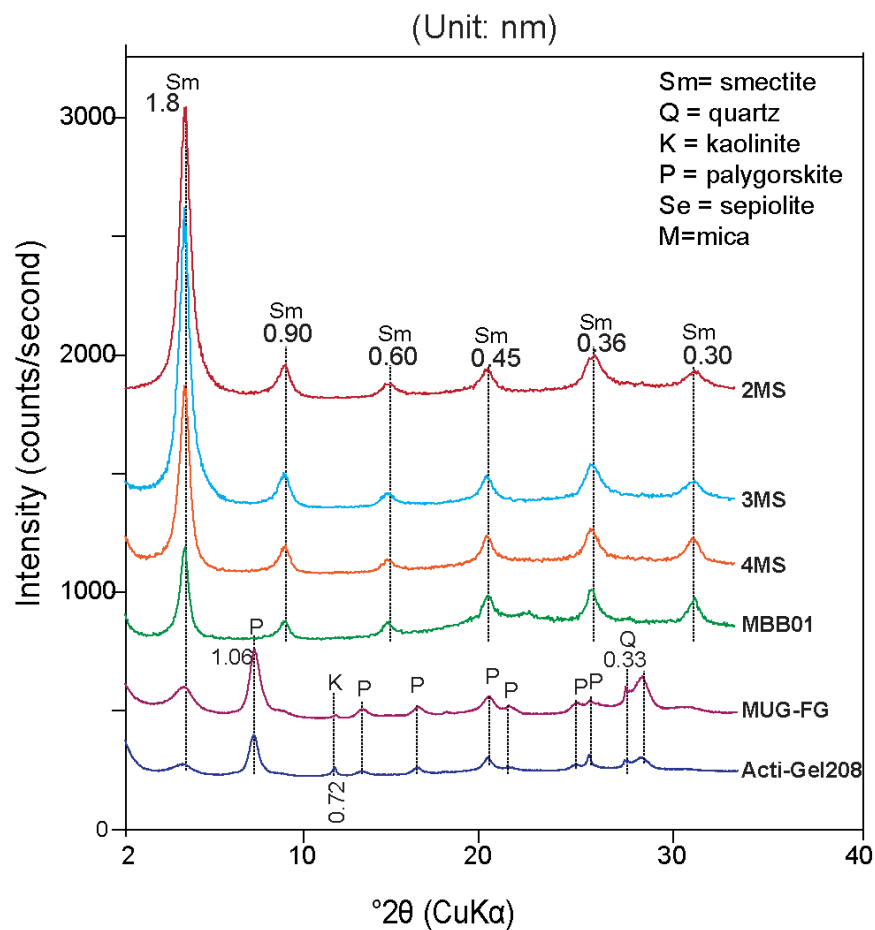


Figure 15. XRD spectra of the Mg-glycerol saturated clays of bentonites and palygorskites.

III.3.3. FTIR analyses

The bulk bentonites showed similar IR spectra except the absence of band 875 cm^{-1} in MBBO1 (Figure 16). The band 912, and 875 cm^{-1} is the reflection of Al_2OH and $\text{Fe}^{3+}\text{-AlOH}$, respectively. The presence of octahedral Fe and amorphous silica in smectite was proved to be responsible for greater aflatoxin B_1 adsorption (Tenorio et al., 2008). The spectra of palygorskites were different. They were characterized by their particular IR bands 1193 and 975 cm^{-1} . They were lack of $\text{Fe}^{3+}\text{-AlOH}$ or AlMgOH .

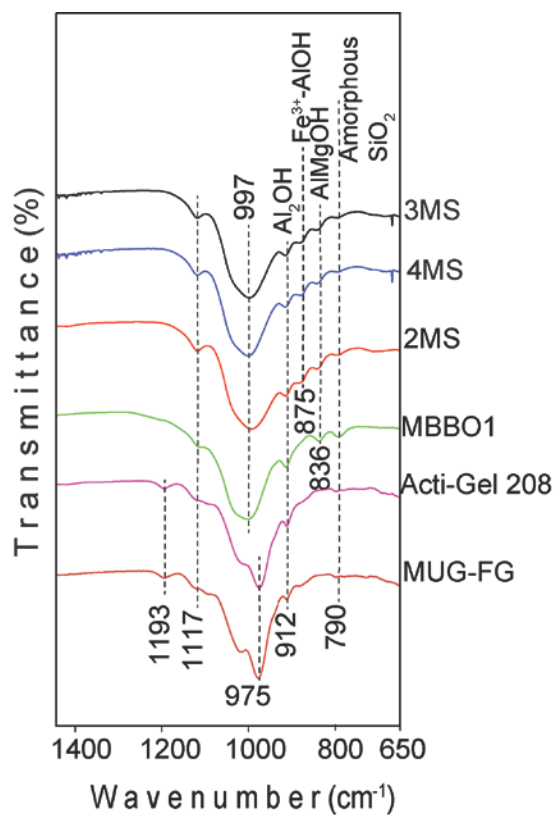


Figure 16. DRIFT spectra of the bulk samples.

The spectra of the clay pellets showed that only MBBO1 did not exhibited the band 879 cm^{-1} , suggesting a very poor presence or complete absence of Fe in the octahedral layer of the this smectite (Figure 17).

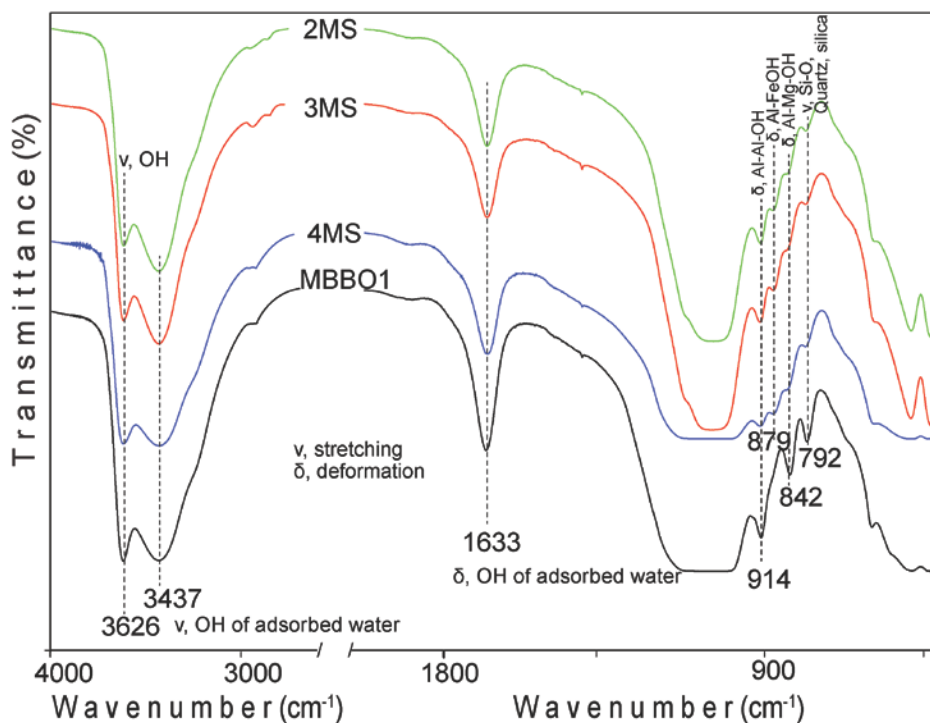


Figure 17. FTIR spectra of the clay pellet of four bentonites.

III.3.4. SEM and TEM of smectites

SEM images revealed that smectites occurred abundantly in all bentonites (Figure 18). The other associated minerals were pyrite, alkali feldspars, quartz, and potassium feldspars. In Acti-Gel 208 and MUG-FG, the dominant mineral palygorskite's fibers were visible with thin flakes of smectites (Figure 19).

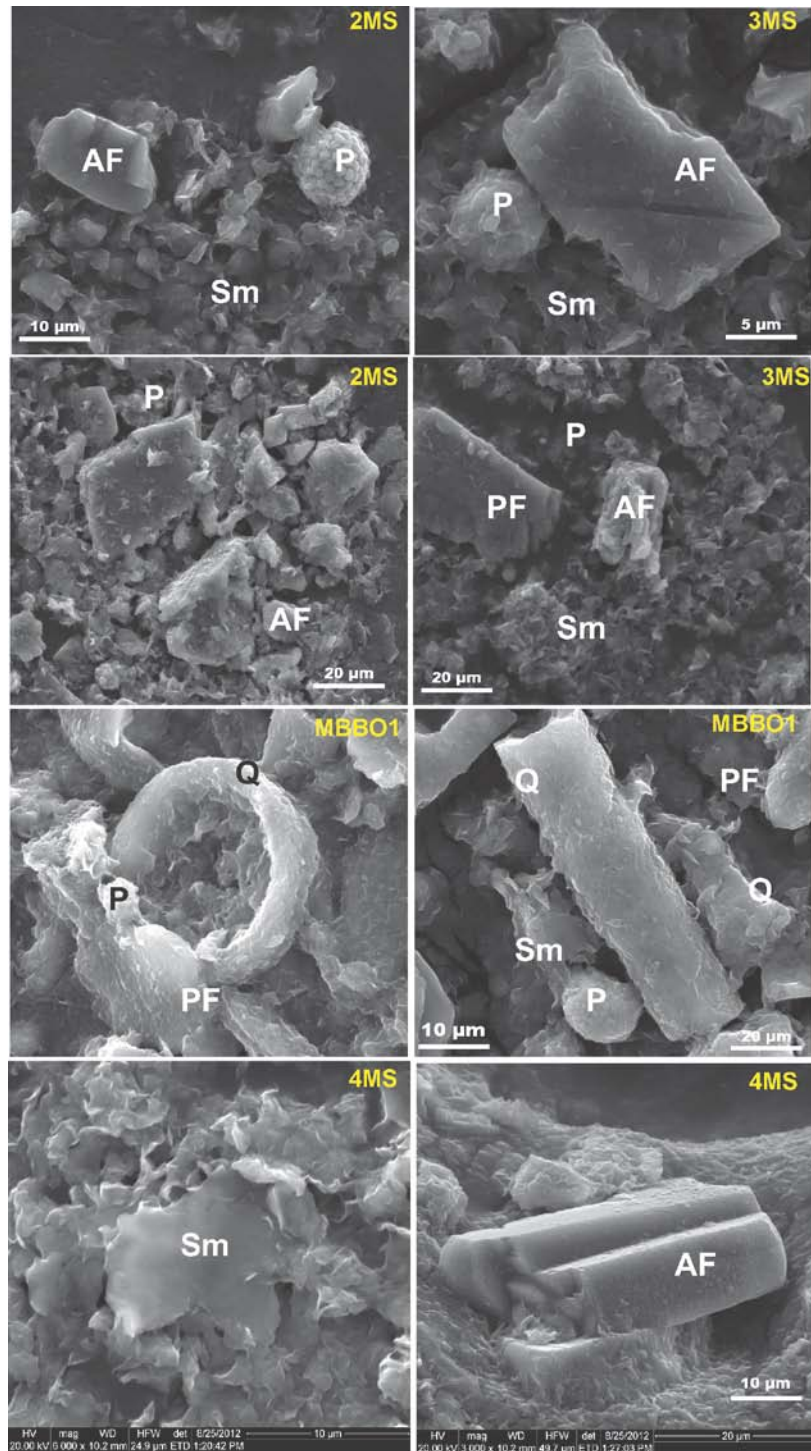


Figure 18. SEM images of smectites associated with other minerals in four southern state's bentonites. Here, Sm=smectite, AF=alkali feldspar, PF=potassium feldspar, P=pyrite, Q= quartz.

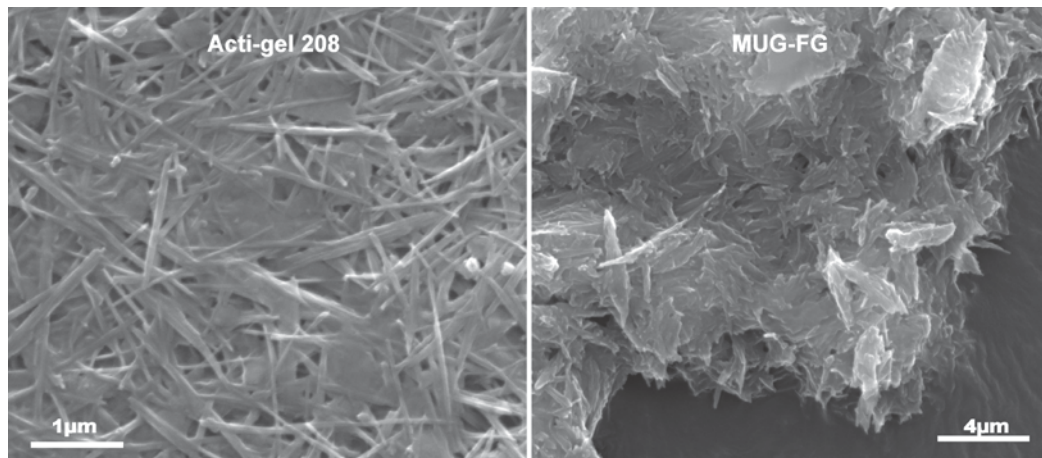


Figure 19. SEM images of the palygorskites.

As all the bentonites showed similar mineralogical composition, TEM analysis was done only for MBBO1. The upper image reflected a good example of typical smectite characterized by very thin layers, wrinkles, folding or fringes visible along its edges, whereas the lower image indicated mixed smectite, characterized by a more complex and diverse granular composition, where the dark scattered spots were due to iron oxides or other heavy minerals (Figure 20).

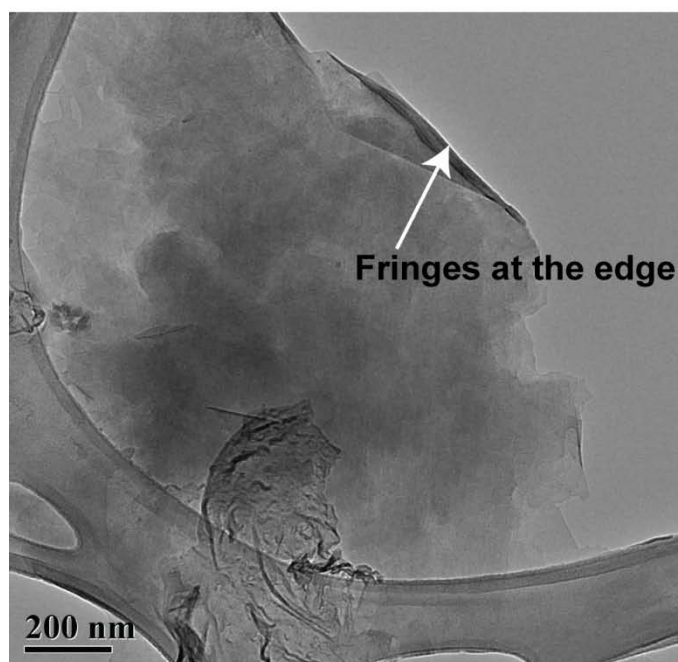
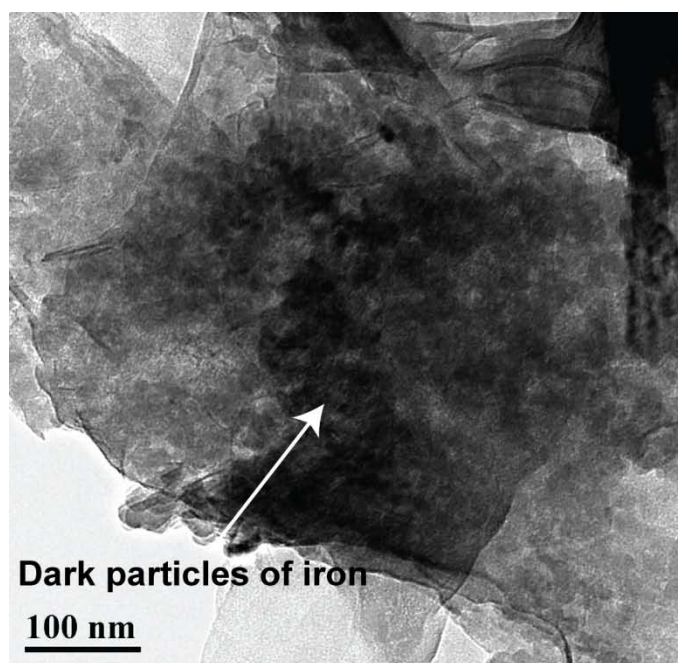


Figure 20. TEM of the clay fraction of MBBO1.

III.3.5. Aflatoxin B₁ adsorption by the smectites in aqueous solution

Langmuir adsorption isotherms of aflatoxin B₁ by the Ca-saturated clays (<2 µm) in aqueous solution suggested that all the bentonites were effective adsorbents as their adsorption capacities, Q_{\max} were more than 0.30 mol kg⁻¹ when checked against the criteria published by Dixon et al. (2008). The isotherm curves were well fitted to the Langmuir model (LM) with showing the L-shape. On the other hand, the palygorskites had very low aflatoxin adsorption capacity (Figure 21).

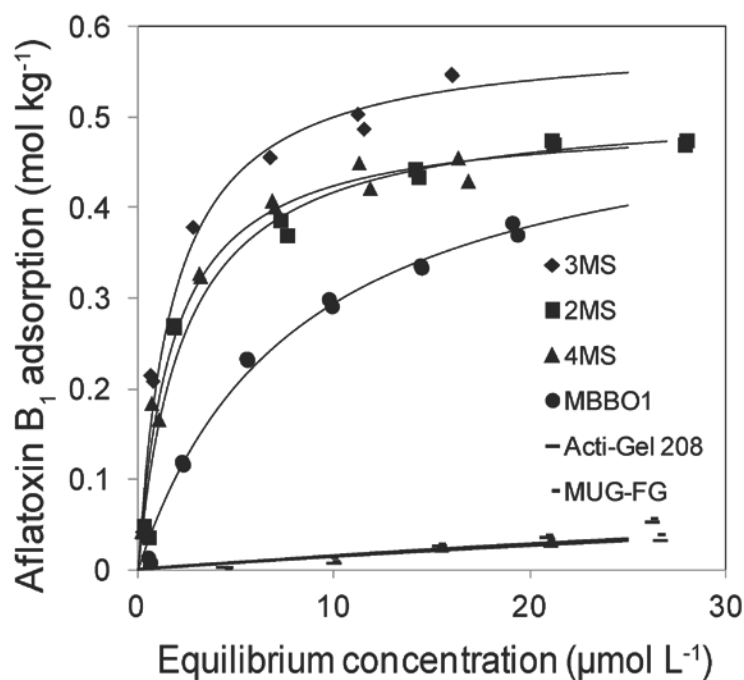


Figure 21. Aflatoxin B₁ adsorption isotherms of the clay fractions (<2 µm) of bentonites and palygorskites at pH 5.5 and 25 °C.

The Q_{\max} varied from 0.36 to 0.50 mol kg⁻¹ among the smectites extracted from four bentonites (Table 3). Maximum adsorption capacity of 0.50 mol kg⁻¹ (15.6% by

clay's dry weight) for 3MS was calculated from the Langmuir equation fit parameter. The comparatively steep slope of 3MS indicated higher affinity of that smectite for sequestering aflatoxin B₁ (Kannevischer et al., 2006). The high aflatoxin adsorption capacity of the bentonites suggested they had high specific surface area. A maximum aflatoxin adsorption capacity of 0.45 mol kg⁻¹ (14% by mass) was found for a Texas smectite which had a specific surface area of 790 m² g⁻¹ (Deng et al., 2010). Although MBBO1 had high adsorption capacity of 0.42 mol kg⁻¹, its adsorption affinity (K_d) was lower than that of 3MS, which gave the lower curve for this smectite (Table 3).

The Acti-Gel 208 and MUG-FG in the present study showed very low aflatoxin B₁ adsorption capacity. Their Q_{max} was 0.038 and 0.037 mol kg⁻¹ (Table 3). The results indicated that the palygorskites were not good candidates for aflatoxin adsorption. The poor aflatoxin adsorption by these clay minerals was likely due to the presence of very low quantity of smectite in them. Despite possessing similar 2:1 layer basic units in palygorskite like in smectite, aflatoxin molecules apparently could not access the tunnels of the palygorskite.

Table 3. Langmuir (LM) adsorption isotherm parameters of the bentonites and palygorskites.

| Samples | AfB ₁ adsorption capacity Q_{\max} (mol Kg ⁻¹) | AfB ₁ adsorption affinity K_d (M ⁻¹) | Weight basis adsorption (%) |
|-------------|--|--|-----------------------------|
| 3MS | 0.50 | 1.02×10^6 | 15.6 |
| 4MS | 0.43 | 0.81×10^6 | 13.4 |
| MBBO1 | 0.42 | 0.22×10^6 | 13.1 |
| 2MS | 0.36 | 1.74×10^6 | 11.2 |
| Actigel 208 | 0.038 | 5.50×10^6 | 1.1 |
| MUG-FG | 0.037 | 4.15×10^6 | 1.0 |

III.4. Conclusions

The present study described the mineralogical analyses of some bentonites and palygorskites, naturally originated in southern states of the U.S. The analyses of the samples identified well saturated smectites in the bentonites. The *in vitro* aflatoxin adsorption experiments revealed the efficiency of the smectites as aflatoxin binders. Results confirmed that all the bentonites were moderately to highly efficient to detoxify aflatoxin B₁ in simplified aqueous solution. Bentonite 3MS showed the best aflatoxin adsorption capacity, whereas the MBBO1 had the lowest adsorption. The lacking of octahedral Fe in MBBO1 structure was the possible reason for comparative lower aflatoxin adsorption of this bentonite. The adsorption capacity of the smectites can be enhanced by modifying their interlayer exchangeable cationic configuration. These studied smectites can be applied as additives in animal feed to mitigate toxicity problem in livestock and human. The palygorskites had very poor aflatoxin adsorption capacity, and were not considered as good agents for further application in detoxification purpose.

CHAPTER IV

MINIMAL INTERFERENCE OF GLUCOSE AND ETHANOL ON AFLATOXIN B₁ ADSORPTION BY SMECTITES *

IV.1. Introduction

Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*, and are carcinogenic to animals and humans (Murphy et al., 2006). A number of foods around the world were detected with high mycotoxin levels, among them corn was considered as the most vulnerable crop to aflatoxin attack especially in droughty regions.

With increasing demand on sustainable energy, about 34% of the corn produced in the U.S. is used for biofuel production (Donner and Kucharik, 2008). Maize contributed more than 80% of the entire production of ethanol fuel (Kelderman, 2007). Mycotoxin contaminated corn that lost their suitability to be consumed as food or feed is randomly utilized in ethanol plants for biofuel production. The co-products of ethanol production or DDG are dried from wet distiller's grain with the concentrated thin stillage to 10–12% moisture. DDG are lower in cost relative to maize and soybean meal. Despite of being highly nutritious, cheap, and widely available to farmers, the acceptance of DDG as animal feed often questionable regarding the three-fold accumulation of mycotoxin compared to the original contaminated corn used during biofuel production.

*Part of the data reported in this chapter is reprinted with permission from “Minimal interference of glucose and ethanol on aflatoxin B₁ adsorption by smectites” by Sabrina Sharmeen Alam, Youjun Deng, and Joe B. Dixon., 2015. *Applied Clay Science*, vol. 104, page 143-149. Copyright© [2015] by Elsevier B. V.

Although a controversy exists whether aflatoxin is degraded or not during fermentation, most of the researchers concluded that aflatoxin accumulated up to three-fold in the ethanol co-products (Bennett and Richard, 1996; Bothast et al., 1992; Schaafsma et al., 2009; Wu and Munkvold, 2008).

Inspired by the fact that some smectites in the previous study showed high aflatoxin B₁ adsorption capacity in aqueous solution, those smectites were evaluated further for their potentiality as aflatoxin inactivating agents during biofuel production.

The various components in the fermentation solution may reduce or completely eliminate the aflatoxin adsorption capacity of smectite. Ethanol and glucose (molecular weight: 46.07 and 180.16 g mol⁻¹, respectively) are the two major soluble compounds in fermentation solution that might be adsorb by clays readily (Dowdy and Mortland, 1968; Pusino et al., 1989). Ethanol and glucose molecules might compete with aflatoxin B₁ for the clay's adsorption sites. Therefore, they could reduce or inhibit aflatoxin adsorption in fermentation solution. Other compounds such as protein, amino acid, lactic acid, acetic acid, phosphorus, and various biopolymers produced during fermentation may also be adsorbed to the smectites, and thus could block potential aflatoxin adsorption sites.

Due to the complex chemical compositions and various interferences from different compounds, it would be difficult to address the effects of all components in fermentation solution on aflatoxin adsorption at once. This report addresses the effects of glucose and ethanol on the adsorption of aflatoxin B₁ by smectites. The specific objectives of this study were: 1) to evaluate the aflatoxin B₁ adsorption capacity of the smectites in the presence of glucose and ethanol up to the maximum concentrations

typically found in fermentation solutions, 2) to verify the interlayer accessibility of smectite for aflatoxin B₁ those two solutions, and 3) to evaluate the influence of glucose and ethanol on the bonding characteristics between aflatoxin B₁ and smectites.

IV.2. Materials and methods

IV.2.1. Smectites studied

In the present study calcium saturated smectite clays previously extracted from bentonite 2MS, 3MS, 4MS, and MBBO1 were used for the overall experiments. The clays were extracted according to the size fractionation procedures from Deng et al. (2012).

IV.2.2. Procedures for aflatoxin B₁ adsorption

The methodology for isothermal adsorption of aflatoxin B₁ was almost same as the one used by Kannewischer et al. (2006) with some changes in composition of solution. Information on source of aflatoxin B₁, stock solution preparation, and preservation was described in detail in the Chapter III.

To simulate fermentation solution, the 8 ppm aflatoxin B₁ working solutions were prepared in 10% glucose (w/v) and 20% ethanol (v/v) solution, respectively. One 8 ppm working solution was also prepared in distilled water. The seven concentrations such as 0.0, 0.4, 1.6, 3.2, 4.8, 6.4, and 8 ppm aflatoxin B₁ solutions were prepared by mixing the 8 ppm aflatoxin B₁ with corresponding blank 10% glucose, 20% ethanol, and 100% aqueous solution, respectively.

IV.2.3. Synthesis of smectite-AfB₁ complexes for FTIR and XRD analyses

To assess if ethanol and glucose would reduce the interlayer accessibility of smectite for aflatoxin B₁, and the bonding between smectite and aflatoxin B₁, smectite-AfB₁ complexes were synthesized in the presence of 10% glucose and 20% ethanol solutions. These complexes were also compared with the smectite-AfB₁ complex, where aflatoxin B₁ was prepared in 100% aqueous solution.

One mg of calcium smectite was added to each tube containing 40 mL of 8 ppm aflatoxin B₁ solution prepared in aqueous, glucose, and ethanol solutions. The smectite-aflatoxin dispersions were shaken for 24 hours at 200 rpm, and centrifuged at 4500 rpm for 57 min. The treatment was repeated one more time by replacing the supernatant with additional 35 mL of aflatoxin B₁ solution with the same chemical composition as the first treatment. The aflatoxin B₁ sequential loadings by smectites were measured at each washing with the UV spectrophotometer. The complexes were washed with distilled water twice to remove excess aflatoxin and compounds not adsorbed by the clays. The clay residues were dispersed in ~0.5 mL water and kept in a refrigerator at 4°C for XRD and FTIR analyses. Two bentonites such as 3MS and MBBO1 were selected for the synthesis of the smectite-AfB₁ complexes. The 3MS was preferred due to its highest aflatoxin adsorption capacity among the all bentonites.

For FTIR spectroscopy analyses, the smectite-AfB₁ complexes were air dried as thin films on 25 mm × 2 mm ZnS discs (ClearTran, International Crystal Labs, Garfield, New Jersey, USA) and mounted in a dewar accessory (model DER-P11-3, Harrick Scientific Products, Inc. Pleasantville, New York, USA). FTIR spectra were recorded in

the transmission mode on a Spectrum 100 Fourier transform infrared spectrometer (Perkin-Elmer) at ~0% humidity by purging dry N₂ gas into the chamber.

For variable temperature XRD analyses, all smectite-AfB₁ complexes on the ZnS discs were re-dispersed with few drops of distilled water and air dried on the polished side of 0.50 mm × 130 mm × 150 mm silicon plates diced from a (100) silicon wafer (University Wafers, Boston, Massachusetts, USA). Each silicon plate was placed on top of the sample cup of a reactor chamber XRK 900 (Anton Paar GmbH, Graz, Austria) and carefully aligned with respect to the goniometer axis of the Bruker D8 Advance X-ray diffractometer. The samples were heated from 50 to 300 °C at a rate of 0.1 °C/S and XRD patterns were recorded at every 50 °C interval. The XRD pattern of one pure smectite was also recorded to compare the d₀₀₁ spacing with that of the smectite-AfB₁ complexes under the same heating treatment.

IV.3. Results and discussions

IV.3.1. Effects of glucose and ethanol on aflatoxin B₁ adsorption by smectites

The Langmuir adsorption isotherms of all the smectites in the presence of 10% glucose and 20% ethanol solutions suggested that these two compounds had negligible interferences on the smectite's adsorption capacities for aflatoxin B₁ (Figure 22).

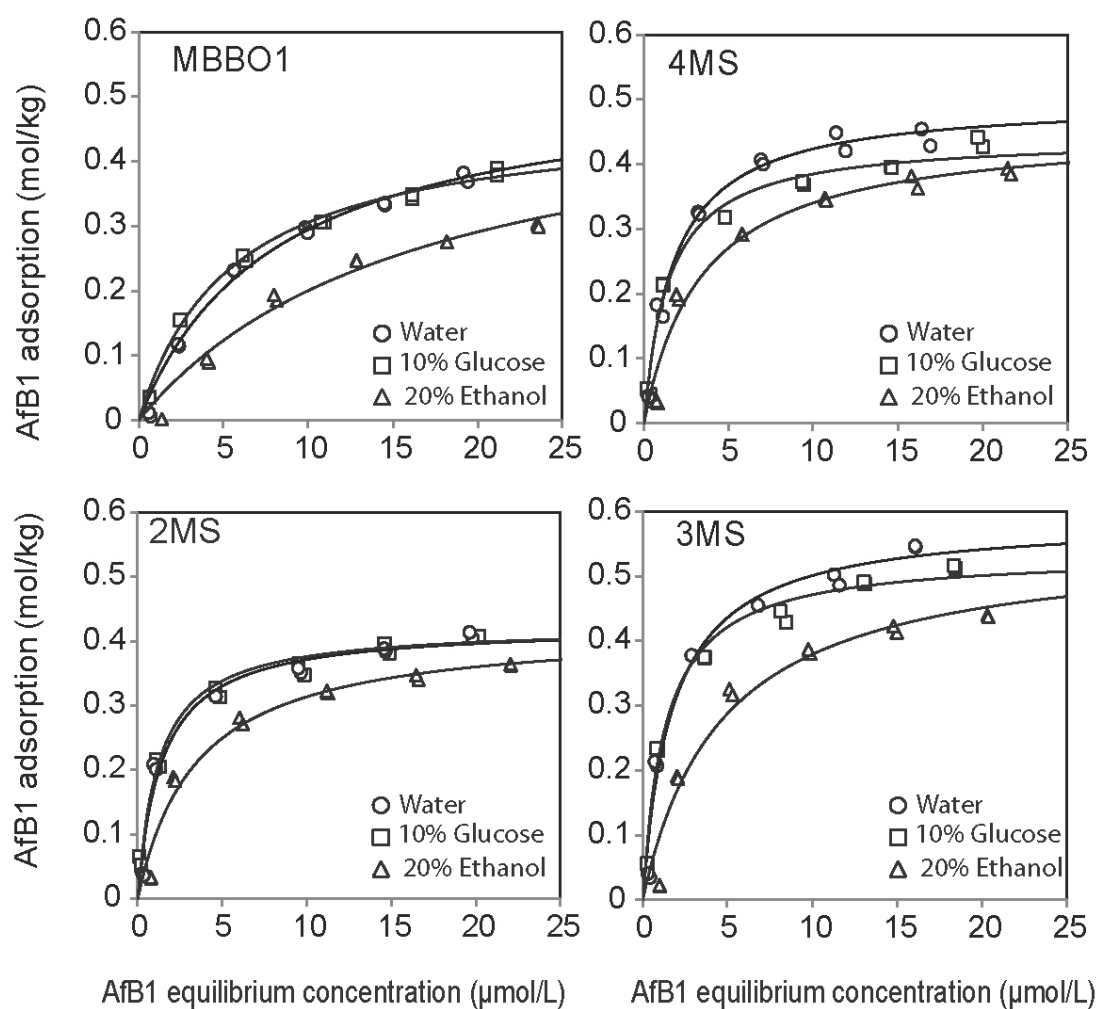


Figure 22. Aflatoxin B₁ adsorption by smectites in aqueous, glucose, and ethanol solutions.

Compared with the adsorption in pure aqueous solution, at least 84% of the smectite's aflatoxin B₁ adsorption capacity of 3MS was preserved even with such high concentrations of ethanol solution (Table 4). The adsorption efficiency by the other smectites in 20% ethanol solutions was even higher than adsorption by 3MS. The reason for sight increased adsorption in 10% glucose solution is a matter of further investigation. However, the adsorption affinity, K_d in both solutions were lower than the affinity in 100% aqueous solution.

Table 4. Comparison of aflatoxin B₁ adsorption characteristics among aqueous, 10% glucose, and 20% ethanol solutions.

| Samples | AfB ₁ adsorption capacity Q_{\max} (mol kg ⁻¹) | | | AfB ₁ adsorption affinity K_d (M ⁻¹) | | | Adsorption efficiency in ethanol based on Q_{\max} (%) |
|---------|--|---------|---------|--|--------------------|--------------------|--|
| | Water | Glucose | Ethanol | Water | Glucose | Ethanol | |
| 3MS | 0.50 | 0.51 | 0.42 | 10.2×10^5 | 7.6×10^5 | 6.59×10^5 | 84 |
| 4MS | 0.43 | 0.52 | 0.39 | 8.09×10^5 | 1.90×10^5 | 4.4×10^5 | 90 |
| MBBO1 | 0.42 | 0.57 | 0.46 | 2.15×10^5 | 0.68×10^5 | 0.67×10^5 | 109 |
| 2MS | 0.36 | 0.49 | 0.36 | 17.4×10^5 | 1.85×10^5 | 4.08×10^5 | 100 |

IV.3.2. Sequential loading of aflatoxin B₁ in the smectite's interlayer during XRD and FTIR sample preparation

During the two sequential treatments of the smectites with aflatoxin solutions, more than 85% of the total aflatoxin B₁ adsorption by the smectites occurred during the first treatment. The presence of glucose or ethanol did not show any influence on this trend (Figure 23). Aflatoxin loading on 3MS reached 0.8 mol kg⁻¹ (27% w/w) in the

absence of the ethanol or glucose. Aflatoxin loading on the MBBO1 was lower than on 3MS. Ethanol and glucose reduced the loading of aflatoxin on the two smectites but the highly efficient smectite clays were still capable to bind aflatoxin B₁ at a rate of minimum 80% of its adsorption capacities while smectite were saturated with 10% glucose and 20% ethanol solutions. Such high loading of aflatoxins on the clays required large surface areas, suggesting the aflatoxins were still able to access the interlayer spaces of smectites.

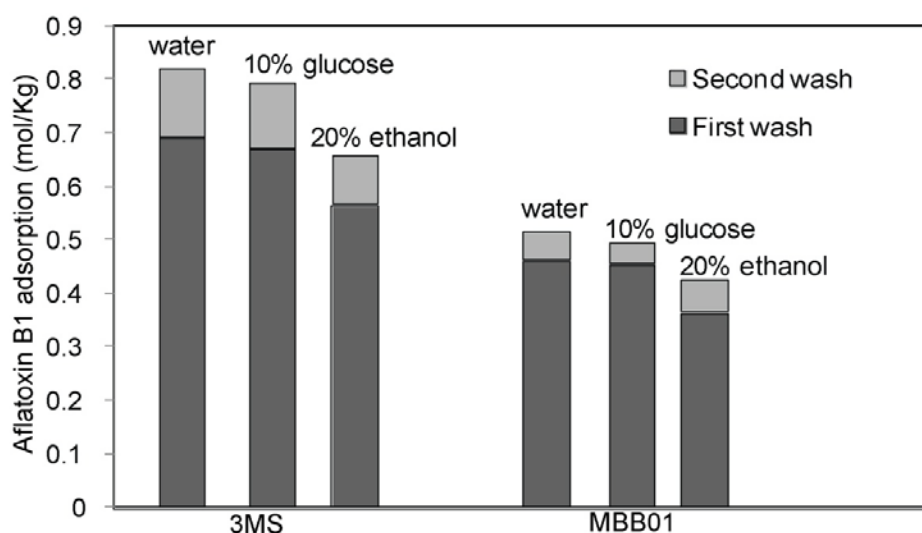


Figure 23. Sequential loading of aflatoxin B₁ in the smectites, measured during sample preparation for XRD and FTIR.

According to the aflatoxin sequential loading experimental graph, the majority loading during the first washing and remarkable adsorption reduction during second washing confirmed nearly complete saturation of aflatoxin B₁ in the interlayer of smectites. The reason for different aflatoxin B₁ adsorption values in the Figure 23 from

the previous Langmuir isotherms adsorption data (Table 4) was most certainly due to following different sample preparation method.

IV.3.3. Effects of glucose and ethanol on interlayer accessibility of smectite for aflatoxin B₁

The variable temperature XRD analysis indicated that the interlayer of smectites was still accessible for aflatoxin B₁ in the presence of high concentration of glucose or ethanol. For example, there was appreciable difference in the d_{001} spacing between the 3MS and 3MS-AfB₁ complexes when heated at elevated temperatures such as >150 °C (Figure 24).

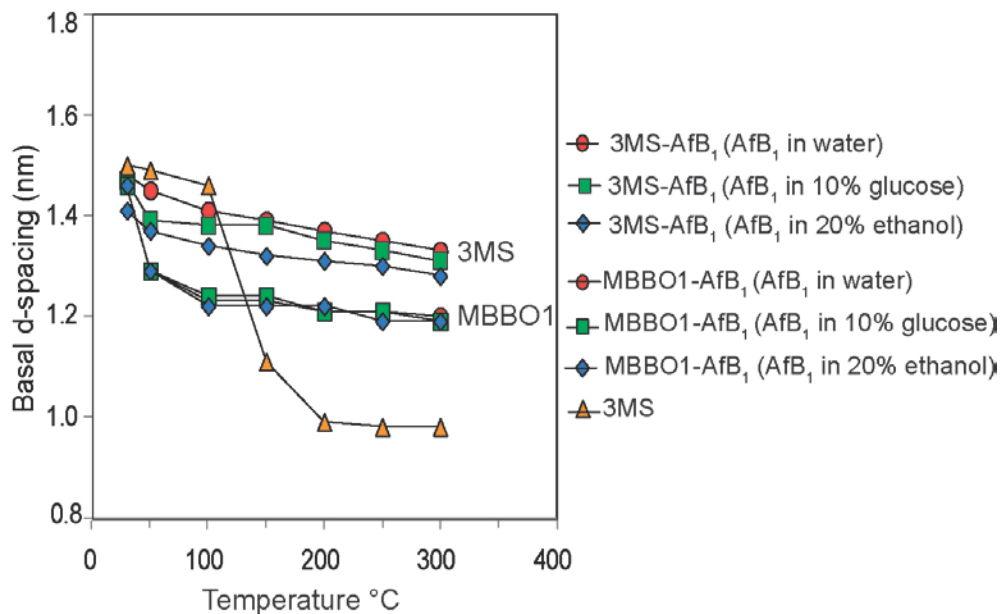


Figure 24. Basal spacing of a pure smectite and smectite-AfB₁ complexes.

Compared with the 3MS-AfB₁ complexes, 3MS had higher d₀₀₁ spacing from 1.5 to 1.46 nm at temperatures from 30 to 100 °C, but a dramatic decrease in the basal spacing to ~1.0 nm was observed at 100 to 150 °C and then the d₀₀₁ spacing of the smectite remained at ~1.0 nm upon heating up to 300 °C. A similar collapse has been observed on other smectites at 150 °C (Deng et al., 2012, 2010). It appeared to be a common response for Ca-smectites when they are heated under this experimental condition and therefore, only one smectite was checked here. Although all smectite-AfB₁ complexes had close d₀₀₁ spacing at 30 °C, variations were observed when heat treatment proceeded. The minimum d₀₀₁ spacing of the smectite-AfB₁ complexes was 1.2 nm when heated up to 300 °C. The d₀₀₁ spacing for the 3MS-AfB₁ complexes was greater than the d₀₀₁ spacing of the MBBO1-AfB₁ complexes. After heating at 300 °C, the basal spacing values of 3MS-AfB₁ complexes formed in 100% aqueous, 10% glucose, and 20% ethanol were 1.33, 1.31, and 1.28 nm, respectively. The very negligibly differences in the d₀₀₁ spacing between the smectite-AfB₁ complexes in ethanol or glucose, and the complexes in pure aqueous solution at the elevated temperature suggested that maximum quantities of aflatoxin molecules could enter the interlayer spaces of the smectites, despite the presence of high concentrations of ethanol and glucose.

IV.3.4. Effects of glucose and ethanol on smectite-AfB₁ bonding revealed by FTIR

For both smectites 3MS and MBBO1, identical IR band's position for aflatoxin B₁ were observed on the smectite-AfB₁ complexes formed in the presence of 10% glucose and 20% ethanol solutions when compared with those bands in smectites-AfB₁ formed in aqueous solution (Figure 25 and 26). Treatment of the smectites with ethanol and glucose solutions did not generated any new band upon reaction with these clay minerals, except a very low intensity band 1744 cm⁻¹ due to 10% glucose saturation.

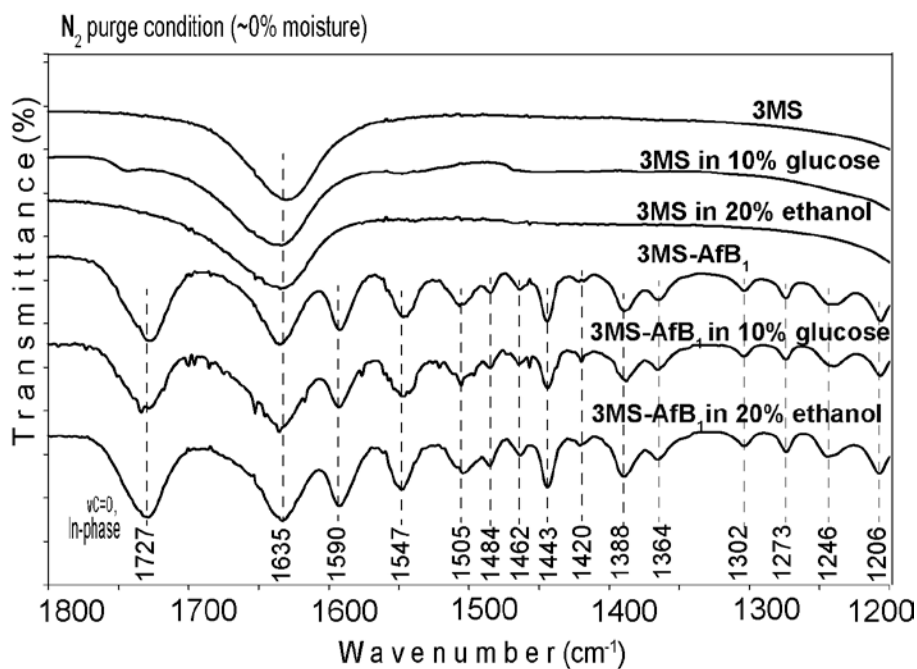


Figure 25. FTIR spectra of 3MS and 3MS-AfB₁ complexes.

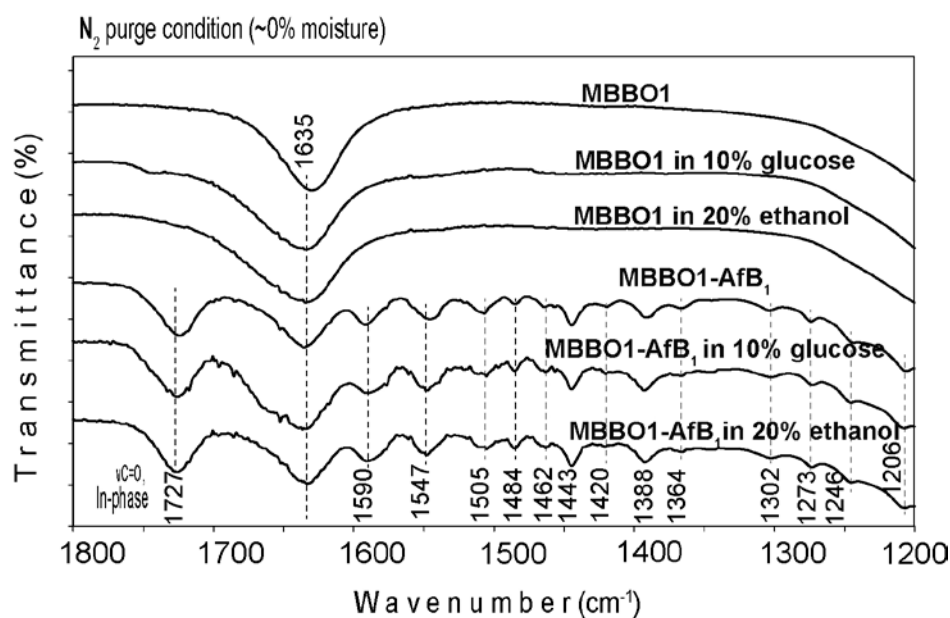


Figure 26. FTIR spectra of MBBO1 and MBBO1-AfB₁ complexes.

The opposite-phase carbonyl stretching vibration occurred at 1635 cm^{-1} and the in-phase carbonyl stretching vibration occurred at 1727 cm^{-1} in all the smectite-AfB₁ complexes. Detail IR assignments and discussion of these bands have been made by Deng et al. (2010). The aflatoxin B₁ band positions were influenced by the types of exchange cations and humidity but not by the charge densities of smectites (Deng et al., 2012). The nearly identical aflatoxin B₁ band positions in all smectite complexes, and no appearance of any additional band due to ethanol or glucose suggested that the smectites were selective for aflatoxins in the presence of these two organic solutions. These two competing compounds did not interfere with the band's position or intensity of the smectite-AfB₁ complexes.

All the bentonites in the present work are considered as good adsorbents because smectites having aflatoxin B₁ adsorption capacity of more than 0.3 mol kg^{-1} were

practically proved as efficient additives in many experiments (Dixon et al., 2008). In this study, only the clay fractions of bentonites were used for adsorption experiments because the sand and silt fractions have extremely poor adsorption capacity compared with the clay fraction due to other accessory minerals. However, some of the bulk bentonites were proved to have greater aflatoxin adsorption capacity than the clay fraction alone due to the large effects of the type of exchange cations on the minerals clay (Mulder et al., 2008).

The intention of this study was not to apply only the clay fraction for mycotoxin remediation during ethanol production. It might not be economically feasible. The purpose for using the calcium saturated clay fractions only was to assess the adsorption capacity of the active mineral such as smectite, and to make a comparable study with the previous researches on aflatoxin–smectite interactions, as most of the works were done based on clay fractions.

A minor decrease in aflatoxin B₁ adsorption by smectites in the presence of glucose and ethanol solutions suggested that these two compounds interfered very weakly with the aflatoxin molecules for clay's adsorption sites. The effects of ethanol and glucose on the smectite's aflatoxin removal efficiency in real corn fermentation solution are expected to be even smaller because the ethanol and glucose concentrations in typical fermentation solutions are less than the upper limits tested in this experiment (Murthy et al., 2005). More than 80% reserving ability of the smectites to bind aflatoxin even in the presence of the upper limit of ethanol suggested that if smectites are applied during the fermentation of corn, there would be minimum interference of ethanol and

glucose on the smectite's ability to adsorb aflatoxin. Yet, the influences of other compounds such as proteins, amino acids, various biopolymers, in the real fermentation solution on the smectite's aflatoxin adsorption should be evaluated.

Literatures suggested that low molecular weight alcohols and sugars can be adsorbed readily by smectites in the interlayer spaces. Therefore, the alcohols and sugars could be strong competitors of aflatoxins for the adsorption sites on smectites. Ethanol adsorption isotherms suggested that more than 0.7 mol kg^{-1} adsorption occurred on smectite yet the adsorption was still below saturation (Zhang et al., 1990). In another study, ethanol was also found to be adsorb into the interlayer of smectites (Comets et al., 1993).

In an infrared spectroscopic study, it was revealed that the adsorption–desorption of ethylene glycol on montmorillonite was a reversible process. Glycol disappeared when the clay–glycol complex was exposed to the atmosphere (Dowdy, R.H. and Mortland, 1968). Desorption of ethanol from montmorillonite was also observed by Pascal et al. (2013). Similarly, single or two layers of sugars could be adsorbed in the interlayer of smectites from aqueous solutions (Greenland, 1956). The adsorption of glucose on clays depends on both the sugar concentration and the nature of the interlayer cation. For example, according to Greenland (1956), maximum adsorption was found in Na-montmorillonite while minimum adsorption occurred in Ca–montmorillonite. His X-ray investigation revealed that 1.45 nm basal d_{001} spacing for the Ca-saturated montmorillonite–glucose complex when the concentration of glucose was half of the clay concentration. Again, basal d_{001} spacing decreased to 1.42 nm when glucose was

less than half of the clay concentration. Despite the high adsorption capacity for the simple alcohols and sugars, it appeared that the adsorbed alcohols and sugars could be readily removed by water washing or evaporation, suggesting that the smectites may not have selectivity for these small organic molecules.

The identical IR spectral positions for the six smectite-AfB₁ complexes in different solutions (Figure 25 and 26) suggested that the adsorbed ethanol or glucose (if any in the complexes) was removed by water washing but the adsorbed aflatoxin B₁ molecules were not removed after repeated washing with water. Once bounded in the interlayer of the smectites, aflatoxin B₁ became almost fixed. The bonding mechanisms between aflatoxin B₁ and smectite were broadly discussed by Deng et al. (2010).

No essential differences in the band position or shape of the smectite-AfB₁ complexes prepared in the aqueous, 10% glucose, and 20% ethanol solutions suggested that there were nearly the same bonding strength existed between the aflatoxin B₁ and smectites in the complexes. Consequently, no remarkable effects of glucose and ethanol were observed on the aflatoxin B₁ adsorption capacities of the smectites.

The higher d₀₀₁ spacing for the 3MS at the temperature from 30 °C to 100 °C indicated the existence of interlayer water, which disappeared and finally the smectite collapsed after heated at 100 °C. The greater d₀₀₁ spacing for the six smectite-AfB₁ complexes compared with pure 3MS at elevated temperatures were due to the irreversible bonding of aflatoxin in the interlayer of all smectites. Aflatoxin B₁ could still access the interlayer of smectite in the presence of such high concentrations of ethanol and glucose. The higher basal spacing of the three 3MS-AfB₁ complexes than the three

MBBO1-AfB₁ complexes under the heating treatment was due to higher adsorption capacity of 3MS than MBBO1. Again, a slight decrease in basal spacing synthesized in the presence of ethanol and glucose suggested that these two compounds had a weak interference on the aflatoxin adsorption by smectites. These two compounds could enter initially into the interlayer of the smectites but were removed after washing with water. The nearly identical d₀₀₁ spacing in the case of three different MBBO1-AfB₁ complexes was most probably due to this smectite's lower affinity for aflatoxin B₁ when compared with those of the 3MS-AfB₁ complexes. It appeared that d₀₀₁ spacing of smectite-AfB₁ complexes were positively correlated with the Q_{max} of the smectites.

IV.4. Conclusions

Smectite's aflatoxin B₁ binding efficiencies in glucose and ethanol solutions were evaluated and the interaction of the two compounds with the smectites was inferred from the mineralogical standpoint. The overall study suggested that ethanol and glucose had minimal interferences on the smectite's efficiency in adsorbing aflatoxin B₁. More than 80% of the smectite's ability to adsorb aflatoxin B₁ was still preserved in the presence of 10% (w/v) glucose and 20% (v/v) ethanol, the upper limit concentrations of these two compounds in real fermentation solutions. Although the adsorption capacity in the two solutions was very close, the affinity of smectites for aflatoxin was relatively low in 20% ethanol, which suggested that glucose had less interference than ethanol on aflatoxin sequestration by smectites. Ethanol and glucose molecules could initially enter the interlayer of smectites but were removed after washing with water. On the other hand, the irreversible bonding of aflatoxin B₁ with smectites occurred in the presence of

the two compounds with high concentrations. The glucose and ethanol compounds did not interfere with the bonding between aflatoxin B₁ and smectites.

The selectivity of smectites for aflatoxin in real corn fermentation solutions will be investigated in further experiment. That experiment will also be aimed to find the major compounds in fermentation solution that might interfere with the aflatoxin B₁ adsorption by smectites.

CHAPTER V

AFLATOXIN B₁ ADSORPTION BY SMECTITES IN CORN FERMENTATION SOLUTION AND POSSIBLE INTERFERING FROM PROTEIN

V.1. Introduction

It has been mentioned that a large quantity of corn in the U.S. is used in biofuel industry for ethanol production (USDA, 2015). To reduce wastage of corn, and toxicity to human and animal, using aflatoxin contaminated corn in biofuel industry is supposed to be rational. Yet up to three-fold of increment of the mycotoxins in the co-products known as dried distiller's grain (DDG) after the fermentation process have detrimental impact on animal health. The economical sustainability of biofuel industries at least partially depends on the marketability of their DDG (Wu and Munkvold, 2008). The ethanol industry as well as the animal industry in close proximity to aflatoxin contamination susceptible regions might have greater concern. Not all the U.S. DDG were detected to have mycotoxin level beyond the FDA regulatory limit (Zhang et al., 2009). Due to three times increment of aflatoxin in DDG, it would be desirable to have the mycotoxins removed, decomposed, or inactivated during the fermentation system.

Ethanol is produced from the corn biomass through the fermentation process with the addition of yeast. Corn fermentation solution is a complex organic solution which typically contains various organic compounds like ethanol, sugar, oil, lipid, lactic acid, acetic acid, glycerol, proteins, etc. In our previous study, we discovered that the two major soluble compounds e.g., ethanol and glucose had minimal interferences on

aflatoxin B₁ adsorption by the smectites (Alam et al., 2015). This discovery led us to expect that smectites would also have high aflatoxin adsorption capacity in real corn fermentation solution. However, the compounds in fermentation solution other than ethanol and glucose, might compete with aflatoxin B₁ for the clay's adsorption sites.

The primary objective of the present study was to evaluate the aflatoxin B₁ adsorption efficiency by smectites in corn fermentation solution. The secondary objective was to identify any interfering compounds that might hinder aflatoxin adsorption.

Maintaining the clay's ability to detoxify aflatoxin from such a complex solution could be a challenge. Question may arise why using clays in corn fermentation solution but not in DDG directly? This is because 1) DDG is a dry material, there would be less chance to have interaction between the aflatoxins and smectites, and 2) on the other hand, aflatoxin in ethanol solution would be more soluble and hence, there would be greater chance of reaction between the mycotoxin and adsorption domains of smectites. However, study showed that addition of sorbents such as brewers dried yeast anti-caking binder containing glucomannon to DDG was found to reduce aflatoxin toxicity significantly compared to positive control (Johnston et al., 2012). The detoxification of aflatoxin during fermentation process is thought to be a more effective strategy to reduce toxicity effects on animals because the toxins are removed or at least reduced to much lower concentration before reaching to DDG. In addition, the consumers would be more interested in clean feed than mycotoxin contaminated feed though treated by clays for detoxification purpose.

The ultimate practical goal of our study was to apply the smectites during biofuel production to reduce the aflatoxins hazards in animal feed from which both the biofuel and animal industries can be benefited. Two questions need to be addressed: (1) what are the major interfering compounds in fermentation solution that might compete with aflatoxin B₁ for clay's adsorption sites?, and (2) how to minimize their interferences and optimize the clay's selectivity for aflatoxin adsorption?. The specific objectives of the current research were: 1) to evaluate aflatoxin adsorption capacity of smectites in corn fermentation solution, 2) to investigate the bonding pattern between aflatoxin B₁ and smectites in fermentation solution, 3) to evaluate whether interlayer aflatoxin adsorption occurred in fermentation solution, and 4) to identify interfering compounds that might block aflatoxin adsorption by smectites.

V.2. Materials and methods

V.2.1. Bentonites used for this study

Five calcium bentonites 3MS and 1MS (Mississippi), MBBO1 (Alabama), 37GR (Greece), and 8TX (Texas) were used for the current study. These bentonites were previously reported as highly efficient aflatoxin binders from aqueous solution (Alam et al., 2015; Deng et al., 2012). Bentonites were size fractionated and the clays (<2 µm) were extracted (Deng et al., 2012). The Na ions of the fractionated clays were exchanged with Ca ions before the aflatoxin adsorption experiment.

V.2.2. Collection of corn fermentation solution (FS)

About six liters of aflatoxin-free corn fermentation solutions containing corn mash were supplied by a local ethanol plant White Energy Inc located at Plainview, Texas in 2013 (Figure 27). The fermentation solutions with corn mash contained ethanol as they were collected before the distillation process. The fermentation process was still going on even after the solutions were stored $\sim 4^{\circ}\text{C}$ in a refrigerator, and CO_2 gas bubbling was observed. Before using in the experiment, the fermentation solution was filtered by the fast flow rate filter paper (15 cm diameter) of Fisher brand®, and then centrifuged to discard any solid materials. Thus, the clean solution was obtained. The pH of fermentation solution was recorded as 4.7. The solution was preserved in the refrigerator for long term use.



Figure 27. A container of fermentation solution mixed with corn mash (collected before the distillation process).

V.2.3. Smectite preparation for FTIR and XRD analyses

To understand if any compound from fermentation solution would adsorb into the interlayer of smectites and thus if they impacted the bonding pattern between smectite and aflatoxin B₁, the smectite complexes for each of 3MS, 8TX, 37GR, MBBO1, and 1MS were prepared in clean fermentation solutions without and with spiked aflatoxin B₁. To compare the adsorption of aflatoxin B₁ in fermentation solution with the adsorption in aqueous solution, smectite-AfB₁ complexes were also prepared in aqueous solution. Therefore, the four samples for each of smectites were (a) pure smectite, (b) smectite-FS, (c) smectite-AfB₁-water, and (d) smectite-AfB₁-FS. The last three complexes were prepared as below:

One mg of Ca-smectite was added to each 50 mL tube containing 35 mL of fermentation or aqueous solution artificially contaminated with 8 ppm aflatoxin B₁. The clay suspensions were shaken for 24 hours at 200 rpm, and centrifuged at 4000 rpm for 57 minutes. The treatment was repeated one more time by replacing the supernatant with additional 30 mL of respective solution. The complexes were washed with de-ionized water twice to remove excess aflatoxins and any substances that were not adsorbed by the clays. The clay residues were dispersed in ~0.5 mL water to avoid drying and kept in refrigerator at 4 °C. Same procedure was followed to saturate the clays in fermentation solution where no aflatoxin was added. The FTIR spectra of the clay films on ZnS discs were recorded in the transmission mode on a Spectrum 100 Fourier transform infrared spectrometer (Perkin-Elmer) at ~0%, ~23%, and ~100% humidity conditions. A dewar accessory was used for FTIR analyses.

For the XRD analyses, samples were analyzed at room temperature (~25 °C) and after heated at 300 °C by XRK900. The instrument information, sample preparation, and analyses procedures for FTIR and XRD were described thoroughly in the Chapter IV.

V.2.4. Procedures for Aflatoxin B₁ adsorption isotherm by smectites in fermentation solution

The procedure for isothermal adsorption of aflatoxin B₁ by smectites was described elsewhere (Kannevischer et al. 2006). The exception was that in this study the 8 ppm aflatoxin B₁ working solution was prepared in diluted fermentation solution (1:3) rather than in simplified 100% aqueous solution. Finally, batch solution containing 0.0, 0.4, 1.6, 3.2, 4.8, 6.4 and 8 ppm aflatoxin B₁ was prepared in the corresponding solutions. For single point aflatoxin adsorption experiment, only 8 ppm aflatoxin B₁ solution was used. The supernatant after adsorption of the mycotoxins by the smectites was analyzed by the Beckman Coulter DU800 UV/visible-spectrophotometer at 365 nm wavelength.

V.2.5. Preparation of smectite-zein complexes

Suspecting that the interfering compound in fermentation solution could be protein, smectite-zein and smectite-AfB₁-zein complexes were prepared following the same procedure described above for XRD and FTIR analyses. Zein is a prolamin protein in corn, which was purchased from Sigma Aldrich. Zein was investigated in its powder form by the ATR (Attenuated Total Reflectance) accessory. To saturate smectites with

zein, zein was dissolved in 90% ethanol (v/v), and distilled water at pH 12 (adding 2 M NaOH). The zein solution was then filtered to remove any particles not dissolved.

V.3. Results and discussions

V.3.1. FTIR investigations of smectite complex in aqueous and corn fermentation solution

The FTIR spectra of the smectite-FS complexes had similar responses to humidity, only MBBO1-FS was shown as an example (Figure 28). Many IR bands appeared were due to the interaction between smectite and the compounds in fermentation solution. These bands were recorded at ~3290, 2926, 2850, 1743, 1652, 1538, 1451, and 1233 cm^{-1} in the range from 4000 to 1200 cm^{-1} . They were absent in the spectrum of pure smectite. The IR band intensities or positions of MBBO1-FS complex did not changed upon humidity variations. Only the bands 1538 and 1233 cm^{-1} at 0% moisture condition shifted very slightly to 1544 and 1237 cm^{-1} , respectively upon 100% moisture treatment.

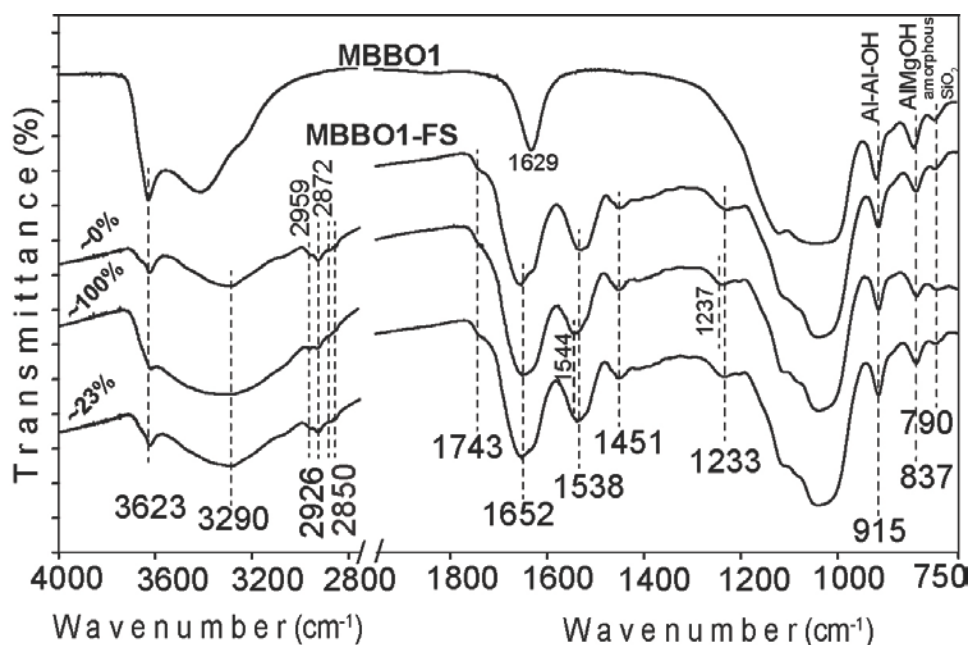


Figure 28. FTIR spectra of smectite and smectite complexes formed in fermentation solution.

As no remarkable variation was observed due to moisture treatment, only the IR spectra recorded at 0% humidity condition for 8TX, 1MS, 37GR, and 3MS treated with fermentation solution were presented (Figure 29). All the smectite-FS complexes showed similar spectra. The similar IR bands that were present in MBBO1-FS complex were also appeared in the four spectra of the four smectite complexes.

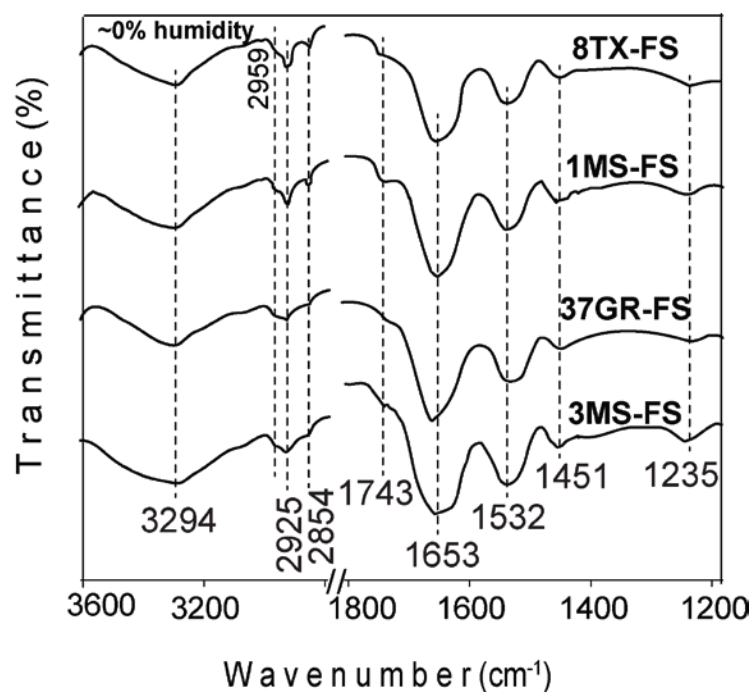


Figure 29. FTIR spectra of smectites after reacting with fermentation solution recorded at ~0% moisture.

Again, the bands were recorded at ~3294, 2925, 2854, 1743, 1653, 1532, 1451, and 1235 cm^{-1} . These bands were completely absent in the respective pure smectites (spectra were not shown). The IR bands at the region between 1800 and 1200 cm^{-1} were known to be the most recorded bands to indicate the bonding between aflatoxin B₁ and smectite. Within this range, the ~1653 and 1532 cm^{-1} were the most intensive and broad bands due to the adsorption of the interfering unknown compounds from fermentation solution in the interlayer of smectites.

FTIR analyses for the five smectites treated in aflatoxin B₁ spiked fermentation solution exhibited the similar IR bands due to interaction between smectite and the compounds from fermentation solution as discussed earlier (Figure 30). The bands were

appeared at 1743, ~1659, 1532/1545, 1443, and 1236 cm^{-1} in the fifteen spectra (three spectra for five smectites). No remarkable differences were noticed in the spectral position or intensity upon moisture variations, except a minor shifting of the band from 1532 to 1545 cm^{-1} when moisture level converted from ~0% to ~100% humidity. Comparison of the IR spectra of smectite-AfB₁-FS complexes with the spectra of smectite-AfB₁ (aflatoxin B₁ in water) complexes revealed that the bands 1595, 1383, 1362, 1304, 1272, and 1205 cm^{-1} in almost all of the smectites appeared because of the adsorption of aflatoxin B₁ molecules in the smectites. It was obvious that the number and intensity of aflatoxin B₁ bands became lower in the smectites-AfB₁-FS complexes compared to the smectites-AfB₁ complexes. More than fifty percent of the aflatoxin B₁ characteristic bands were absent in the smectite-AfB₁-FS complexes compared with the smectite-AfB₁ complexes. This suggested comparatively lower amounts of aflatoxin B₁ were sequestered by the smectites in the fermentation solutions.

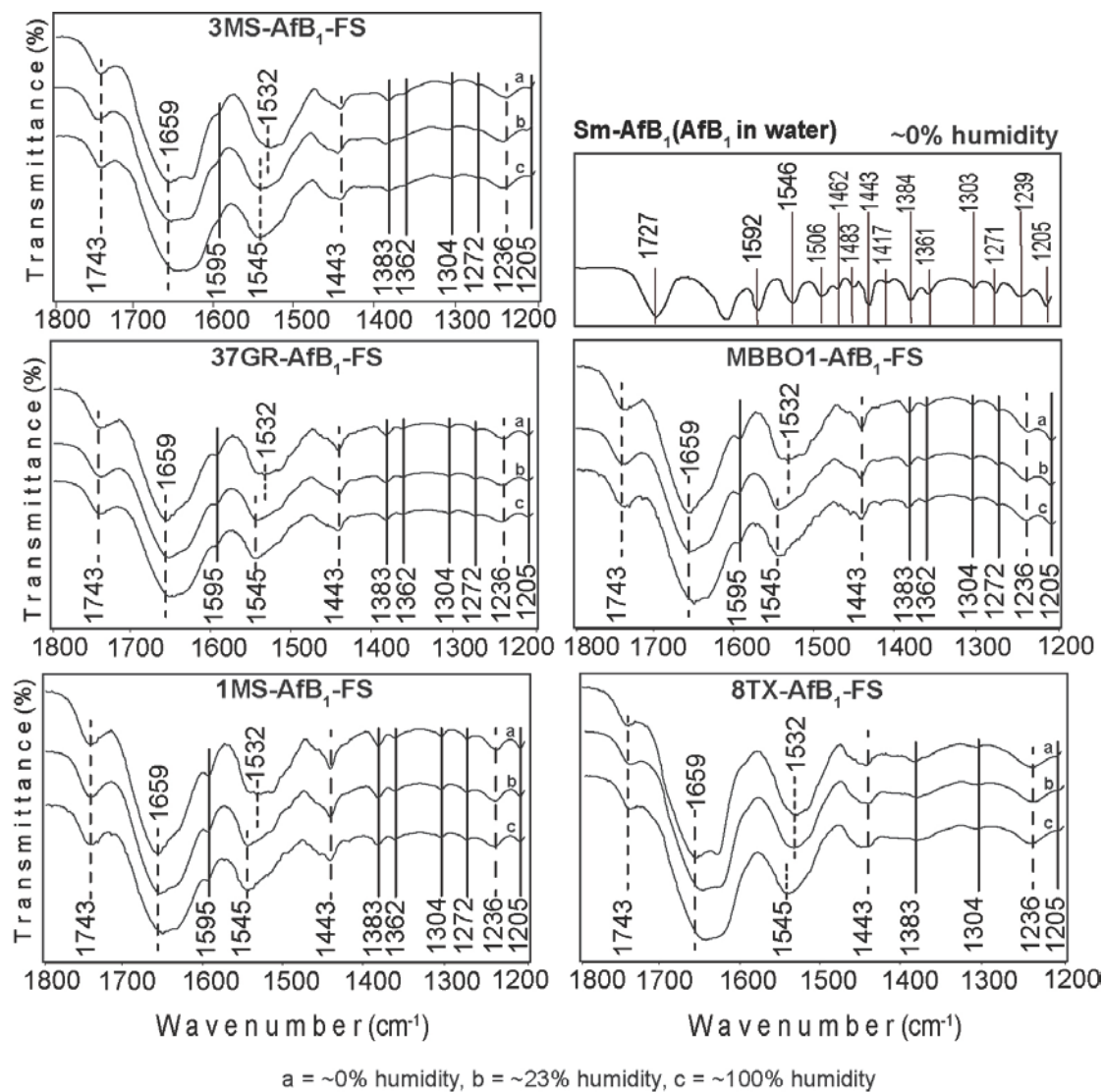


Figure 30. FTIR spectra of smectite complexes under three moisture conditions. Solid lines are the AfB₁ bands.

V.3.2. XRD analyses revealed smectite's interlayer adsorption of compounds from fermentation solution

The interlayer d_{001} spacing of the five pure smectites and their complexes treated after aflatoxin B₁ spiked fermentation solution, pure fermentation solution, and aflatoxin B₁ spiked fermentation solution are presented below (Figure 31). The samples were recorded both at room temperature and 300 °C.

The basal d_{001} spacing for the five pure smectites at room temperature varied from 1.37 nm (8TX) to 1.52 nm (3MS), however, all of them collapsed to ~1.0 nm after heating at 300 °C. On the other hand, the d_{001} spacing of the smectite-FS or smectite-AfB₁-FS complexes increased largely when they were treated in fermentation solutions. For example, the d_{001} spacing of 3MS expanded from 1.52 to 1.82 nm, of 37GR from 1.42 to 1.78 nm, of 8TX from 1.37 to 1.77 nm, of MBBO1 from 1.42 to 1.81 nm, and of 1MS from 1.47 to 1.8 nm at room temperature after treated in fermentation solution. After heating at 300 °C, d_{001} spacing of the same smectites after treatment with fermentation solutions remained up to 1.48, 1.52, 1.50, 1.48, and 1.59 nm, respectively. These d values were even higher than the d values of all smectite-AfB₁ (aflatoxin B₁ in water) complexes.

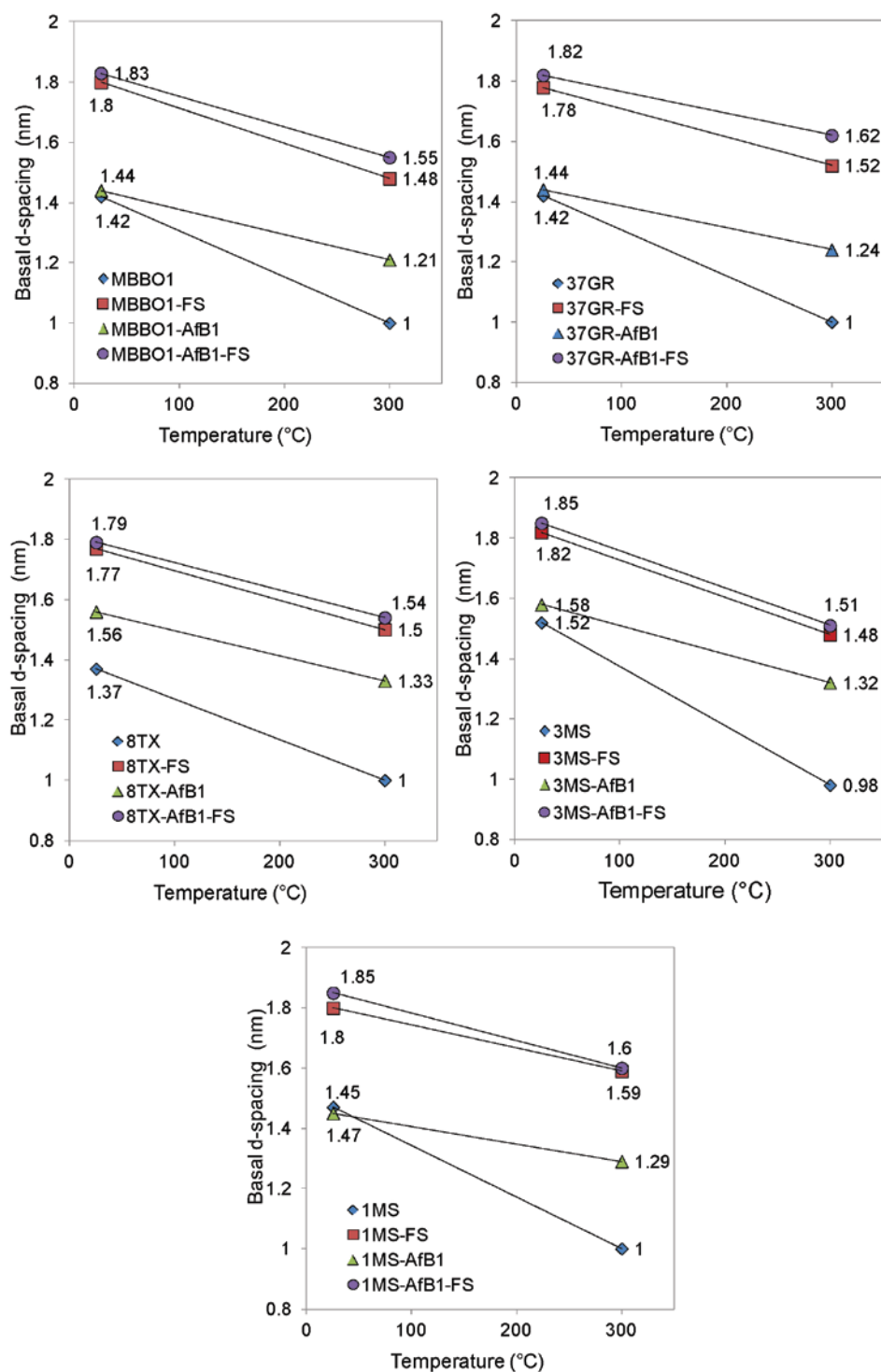


Figure 31. Basal d_{001} spacing of smectites and their complexes in different solutions recorded by thermal XRD (XRD 900).

Though at room temperature, the d_{001} spacing were very close or similar between the smectites and smectite-AfB₁ (aflatoxin B₁ in water) complexes, remarkably higher d_{001} spacing between 1.21 (MBBO1) and 1.33 nm (8TX) were recorded after heating treatment for each smectite-AfB₁ complexes. Negligible differences in the d_{001} spacing, for example, from 1.48 to 1.51 nm, 1.52 to 1.62 nm, 1.50 to 1.54 nm, and 1.48 to 1.55 nm for 3MS, 37GR, 8TX, and MBBO1, respectively were observed between the smectite-FS and smectite-AfB₁-FS complexes after the 300 °C heating treatment. No difference e.g., 1.59 to 1.6 nm was found in case of 1MS.

As XRD and FTIR analyses indicated that due to strong interlayer adsorption of one or more organic compounds from fermentation solution in the smectites, the aflatoxin B₁ adsorption capacity of the smectites in the presence of such complex solutions is supposed to be low.

V.3.3. Aflatoxin B₁ adsorption of smectites in aqueous and fermentation solution

Pilot test was conducted to investigate aflatoxin B₁ adsorption on the smectites in fermentation solution, and was compared with the adsorption in aqueous solution.

As the UV absorbance of undiluted fermentation solution was too high, the solution was diluted to 1:3 (fermentation solution:water) ratio. The diluted fermentation solution yielded a manageable spectrum of aflatoxin B₁ that could be used to quantify aflatoxin B₁ with the UV method (Figure 32).

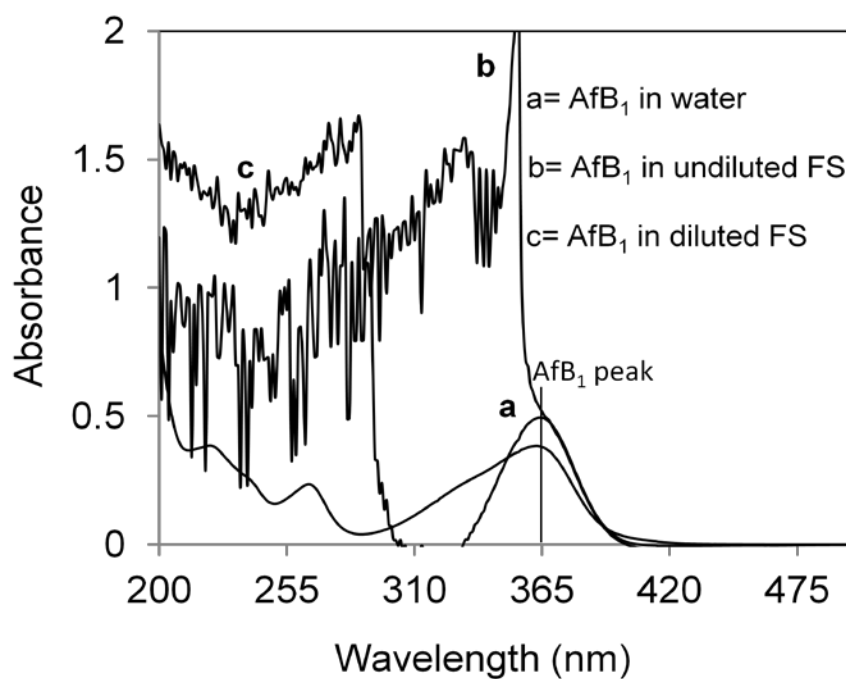


Figure 32. The UV spectra of AfB₁: (a) in aqueous solution, (b) in the undiluted fermentation solution, and (c) in the diluted fermentation solutions (1:3). The peak for aflatoxin B₁ at 365 nm was observed for both a and c.

The standard curves for the aflatoxin B₁ solutions showed that both for corrected and uncorrected form of absorbance, there was a liner relationship ($R^2 = 0.999$) between the absorbance at 365 nm wavelength and the concentrations of aflatoxin B₁ solution (Figure 33).

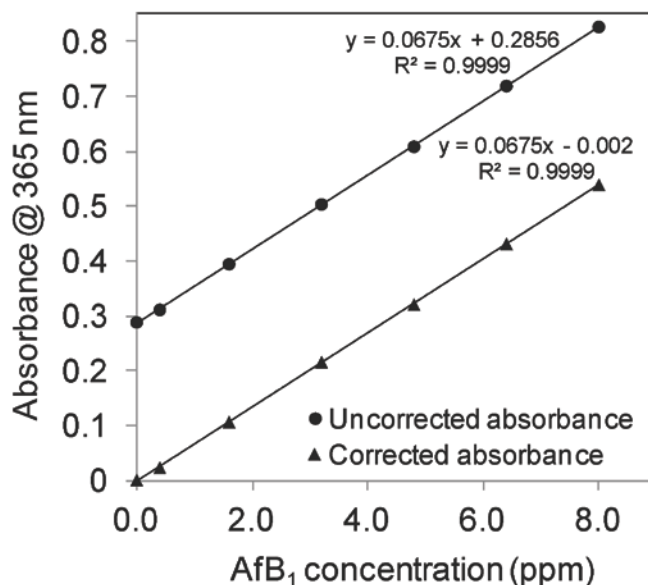


Figure 33. Standard curves of AfB₁ in diluted fermentation solution. The corrected form indicated the subtraction of absorbance due to fermentation solution (0 ppm AfB₁) from each point of AfB₁ concentration.

Due to the similar responses of the smectites to the fermentation solutions, only 3MS was used for the isothermal aflatoxin adsorption experiment.

The aflatoxin B₁ adsorption was lower in the diluted fermentation solution compared to the adsorption by the same smectite in aqueous solution (Figure 34). The adsorption of aflatoxin B₁ in fermentation solution was even remarkably lower than the adsorption in 10% glucose (w/v) and 20% ethanol (v/v) as described in our previous study (Alam et al., 2015).

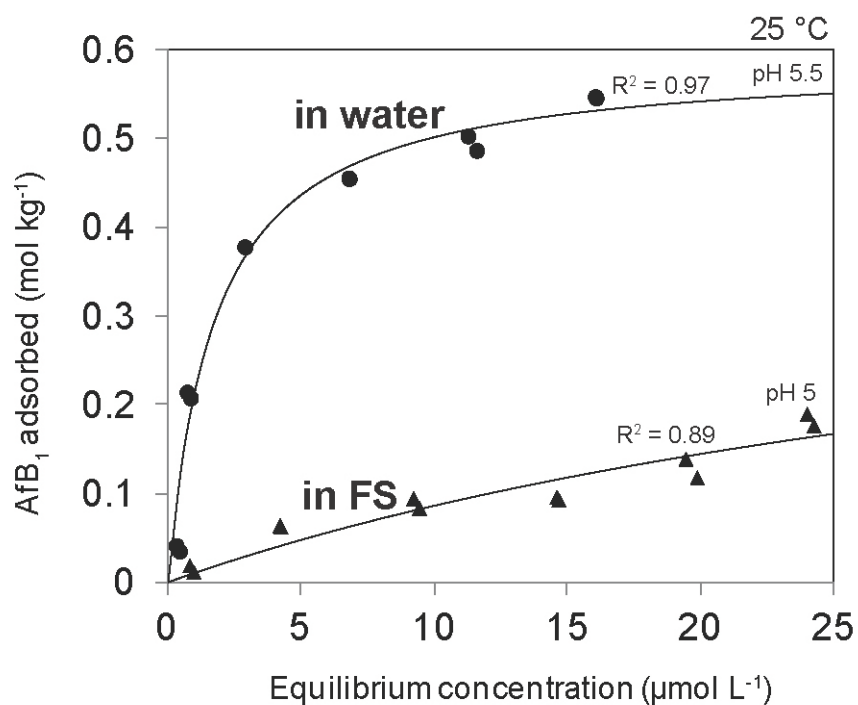


Figure 34. AFB₁ adsorption isotherms of smectite in aqueous and fermentation solution.

The isotherm adsorption curves in aqueous solution and fermentation solution was fitted using the Langmuir equation (LM). Both the aflatoxin B₁ adsorption capacity and affinity by smectite was much lower in fermentation solution than that of in aqueous solution (Table 5). The adsorption capacity, Q_{\max} was decreased from 0.50 mol kg⁻¹ in aqueous solution to 0.209 mol kg⁻¹ in fermentation solution. The adsorption affinity was also declined drastically in fermentation solution.

Table 5. Aflatoxin B₁ adsorption of 3MS in aqueous and fermentation solution.

| Media | AfB ₁ adsorption parameters, Langmuir model (LM) | | Adsorption reduced (%) |
|--------------------------|--|-----------------------------------|------------------------------|
| | Q _{max} (mol kg ⁻¹) | K _d (M ⁻¹) | |
| Aqueous solution | 0.50 | 1.02 × 10 ⁶ | ~58 |
| Fermentation solution | 0.209 | 0.13 × 10 ⁶ | |

The smectites 8TX, 37GR, MBBO1, and 1MS were tested for their adsorption potentiality by considering single point concentration (8 ppm aflatoxin B₁) in the adsorption experiment (Figure 35). Results showed that for each of the smectites, the amount of aflatoxin B₁ adsorbed in fermentation solution was considerably lower than the adsorption in aqueous solution.

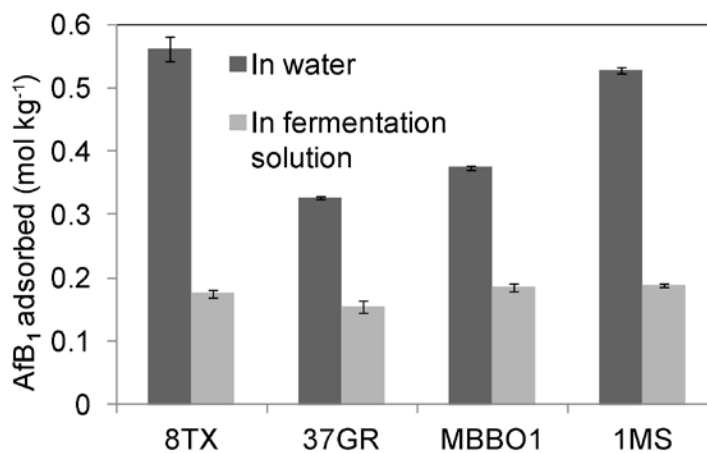


Figure 35. Single point adsorption of aflatoxin B₁ (8 ppm) by four smectites in aqueous and fermentation solution.

V.3.4. Predicting the interfering compounds in fermentation solution by FTIR analyses of zein and smectite-zein complexes

Zein is the major protein in corn and soluble in ethanol. As the fermentation solution contains an appreciable quantity of ethanol, some zein proteins could be expected in the fermentation solution. The presence of the bands 1744, 1646, 1521, 1456, and 1235 cm^{-1} in the solid zein were very similar to the bands for interfering compounds appeared in the smectite-FS complexes (Figure 36).

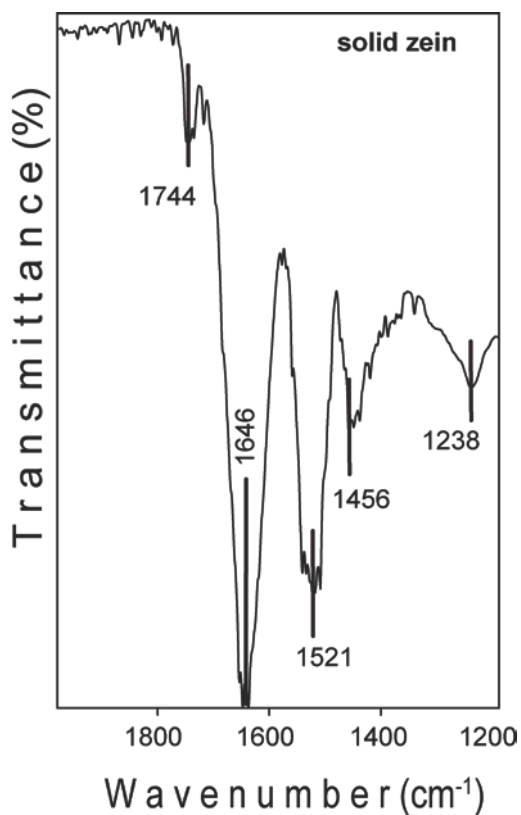


Figure 36. ATR spectra of powder form of zein.

Almost similar position and intensity of the interfering bands such as 1744/1739, 1656/1622/1652, 1530/1538, 1443/1447, and 1235 cm^{-1} were obtained in the IR spectra of one smectite-AfB₁-FS, and two smectite-AfB₁-zein complexes (Figure 37), irrespective of the nature of the solution where zein was dissolved (in ethanol or pH 12 water). Study showed that alcohol solubilization of α zeins did not affect its conformation (Forato et al., 2003). Characteristic aflatoxin B₁ bands were even lesser in smectite-AfB₁-zein complexes than the smectite-AfB₁-FS complex.

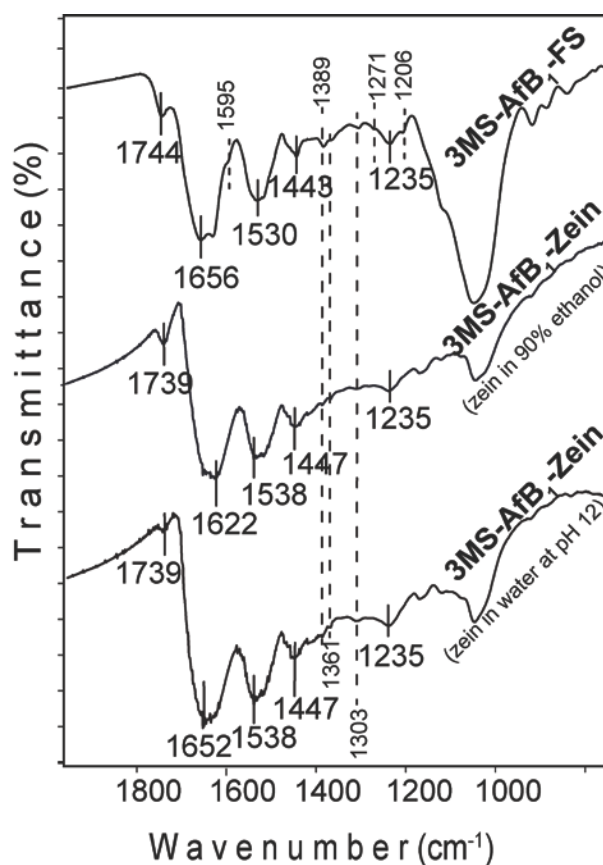


Figure 37. Dewar-IR spectra of smectite-AfB₁ complexes. The solid lines are bands for compounds from zein or fermentation solution, and the dotted lines are AfB₁ bands.

V.3.5. The thermal XRD analyses of smectite and smectite-zein complex

High temperature XRD analysis of the 3MS-zein complex (zein dissolved in 90% ethanol) revealed that the corn protein was adsorbed in the interlayer of smectite (Figure 38). At room temperature, the smectite treated in very high concentration of ethanol (90%, v/v) had d_{001} spacing of 1.52 nm but expanded up to 1.69 nm after treating in ~3% zein solution. Again, smectite collapsed to 0.97 nm after heating at 300 °C but the smectite-zein complex did not collapsed, rather had higher d_{001} value of 1.31 nm.

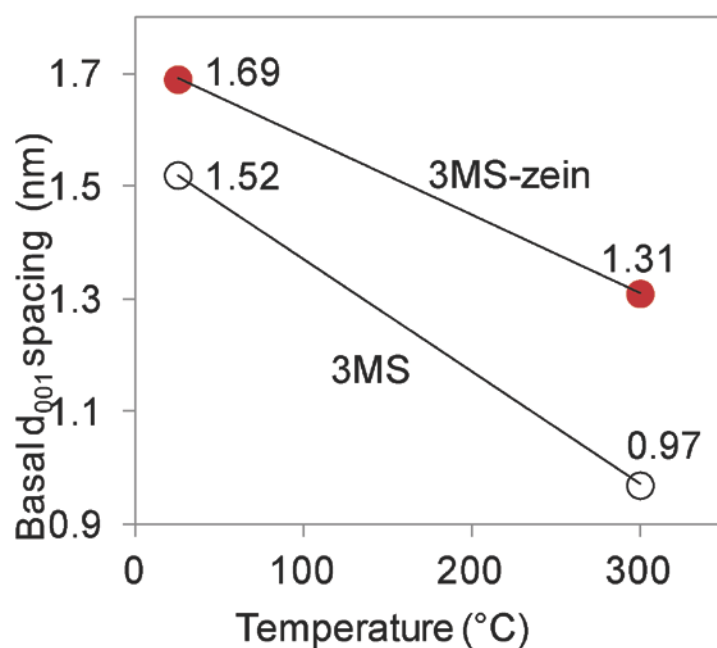


Figure 38. The d_{001} spacing of 3MS after treated in 90% ethanol (lower line) and 3MS after treated in zein dissolved in 90% ethanol (upper line).

The adsorption data of the present study demonstrated that aflatoxin adsorption capacity of smectites in corn fermentation solution reduced at least 50% compared to the adsorption from aqueous solution. This suggested strong interferences of some compounds from fermentation solution that competed greatly with aflatoxin B₁ molecules to occupy the adsorbing sites of the clays, and thus drastically impeded interlayer access of aflatoxin B₁. A study showed that aflatoxin B₁ adsorption capacity of smectite from corn meal extracted by 60% methanol was also found to be significantly lower than the adsorption from water (Jaynes et al., 2007). Another study showed that aflatoxin B₁ adsorption by a Ca-smectite in simulated gastric fluid was very low due to the intercalation of pepsin i.e., protein on smectite (Barrientos-Velázquez et al., 2016), where they also showed the Na-smectite adsorbed more pepsin than the Ca-smectite.

The overall FTIR investigations of the smectite-FS complexes suggested that some compounds from fermentation solution entered the interlayer of smectites. The adsorption of those interfering compounds by the smectites reduced the chance of bonding between aflatoxin B₁ and smectites as indicated by the reduced number of characteristic aflatoxin B₁ bands as well as their low intensities. Early research showed that band 1742 cm⁻¹ was a characteristic band of aflatoxin B₁ in Ba saturated smectite-AfB₁ complexes when treated under 100% humidity but later shifted to 1725 cm⁻¹ when the samples were analyzed under ~0% moisture (Deng et al., 2012). Again, in another study it was observed that band 1743 cm⁻¹ shifted much to lower band when the moisture percentage of the IR chamber changed from ~100 to ~0%, irrespective of the exchange cations (Deng et al., 2010). On the contrary, in the present study this band did not

change or disappeared upon humidity variation. The presence of the minor band 1743 cm^{-1} in all smectite-FS complexes revealed that it appeared due to adsorption of compound, most likely lipids from fermentation solution. The specific IR bands such as ~ 1744 , ~ 1653 , ~ 1532 , ~ 1451 , and $\sim 1235 \text{ cm}^{-1}$ that were generated due to reacting the smectites with fermentation solution, were not found in our previous studies where smectites-AfB₁ complexes were treated in high concentrations of glucose or ethanol solutions (Alam et al., 2015). The current investigations confirmed that those particular five bands were not due to the adsorption of ethanol or glucose from the fermentation solution but were due to the adsorption of other organic compounds by the smectites. The presence of the bands due to the adsorption of interfering compounds on the smectite-FS or smectite-AfB₁-FS spectra even after repeated washing of the clay residues with distilled water suggested that the compounds from fermentation solution had strong attraction to the smectites. The persistence of the compounds on smectite's interlayer suggested they were either positively charged, had great molecular weight, or both. It seemed to be difficult to dissociate those compounds from the clay. Any strategy that block the adsorption of the interfering compounds by the smectites might indirectly reduce their interferences on aflatoxin B₁ adsorption.

In the current study, the characteristic bands appeared due to interaction of interfering compounds on smectite were most probably resulted from intercalation of protein or protein like large organic molecules. Similar IR bands were recognized as bands of protein substances in many other studies. For example, a medium intensity band in the 3300 to 3000 cm^{-1} region was reported due to intermolecular hydrogen

bonding of primary amines (both aliphatic and aromatic) and secondary amines exhibited a single band in the 3450 to 3310 cm^{-1} region (Conley, 1966). In our study, the $\sim 3300 \text{ cm}^{-1}$ could be such band from fermentation solution that had medium intensity and possibly originated as a result of nitrogen-hydrogen (N-H) stretching vibration. The C-H stretching vibrations showed a relatively large contribution of CH_3 groups (2959 and 2872 cm^{-1}) as typical for proteins.

Again, the imino group was generally confirmed by the weakly absorbing C=N stretching vibration from 1690 to 1640 cm^{-1} . Interestingly, the most intensive and broad band $\sim 1656 \text{ cm}^{-1}$ on the smectite after reacting in fermentation solution appeared within this region. It should be mentioned that this region was also recognized as carbonyl (C=O) stretching bands. Differentiation could be made as the C=O stretching bands were more intense than the C=N stretching bands. Furthermore, the bands in the region from 1580 to 1490 cm^{-1} were considered as the bending vibrations of single N-H group of secondary amines. The second broad band $\sim 1532 \text{ cm}^{-1}$ in the smectite-FS complexes appeared within this wave number. A review article mentioned that the N-H stretching vibration gave rise to the amide A band from 3310 to 3270 cm^{-1} , and a weak amide B band from 3100 to 3030 cm^{-1} (Barth, 2007).

In the present study, band $\sim 3294 \text{ cm}^{-1}$ most probably indicated the presence of amino groups in proteins from fermentation solution. It was also discussed by Barth (2007) that band ~ 1650 , ~ 1550 , and from ~ 1400 to 1200 cm^{-1} were due to the presence of proteins such as amide I, amide II, and amide III vibrations, respectively. The bands for water absorption in the mid-infrared spectral region such as 1629 cm^{-1} was

overlapped the broad and intensive amide I band of proteins recorded at $\sim 1652\text{ cm}^{-1}$. Again, in a different study band 1653 and 1530 cm^{-1} were suggested for amide I and amide II, respectively (Sepelyak et al., 1984). The band 1232 cm^{-1} might be due to DNA and RNA coming from the residue of fermentation solution that was mixed with clay.

The XRD analyses of the smectite-FS complexes revealed that compounds from fermentation solution went into the interlayer of smectites, and were adsorbed in large amounts. This was proved by the remarkably greater d_{001} spacing of the five smectite-FS complexes than the d_{001} spacing of pure smectites both at low and high temperatures. Even after heating at $300\text{ }^{\circ}\text{C}$, the d_{001} spacing up to 1.6 nm of the smectite-FS complexes suggested that the interfering compounds were not decomposed fully by heating. The noticeably high d_{001} spacing of the clay complexes after repeated washing again reflected the strong interlayer adsorption of the compounds from fermentation solution. The relatively higher d_{001} spacing of smectite-FS complexes than that of smectite-AfB₁ (AfB₁ in water) complexes demonstrated that the biological molecules such as proteins from fermentation solution were much more potential than the aflatoxin B₁ molecules to occupy the interlayer spaces of smectites, and thus they did interfere aflatoxin B₁ adsorption.

A negligible increase in basal spacing of smectite-AfB₁-FS complexes relative to smectite-FS complexes indicated a very low interlayer adsorption of aflatoxin B₁ into the smectites in fermentation solution. The XRD result was in accordance with the FTIR investigations where very few aflatoxin B₁ bands were present in the smectite-AfB₁-FS complexes. On the other hand, remarkably higher basal spacing in smectite-AfB₁-FS

than the smectite-AfB₁ (AfB₁ in water) complexes was again attributed due to the intensive interlayer access of substances from fermentation solution but surely along with some aflatoxin B₁ adsorption.

Very similar bands position and intensities between the spectra of smectite-AfB₁-zein and smectite-AfB₁-FS indicated that the interfering compounds in fermentation solution were most likely the dissolved protein. The major constituents of corn include starch, protein, oil, and fiber; where protein comprises about 9-12% (w/w) of corn kernel (Earle, 1977). Study showed that protein content of ground corn (~7.5%) increased more than three times (~28%) in the fermented mass produced in three different dry-grind ethanol plants, and remained almost unchanged in dried distiller's grain with soluble (Han and Liu, 2010).

The XRD result (Figure 38) also revealed that the proteins from zein could be adsorbed in the smectite. The greater d_{001} spacing of the 3MS-FS (1.48 nm) than the 3MS-Zein (1.307 nm) indicated a greater adsorption of the compounds from fermentation solution. Therefore, there might be some other biological compounds in such organic complex solution, which also reduced aflatoxin B₁ adsorption of smectites.

The presence and persistence of the proteins in the smectites revealed that they were nonionic or cationic in charge properties, and possibly had irreversible bonding with smectites. Study showed that the adsorption of cationic and nonionic polymers on smectites was irreversible (Deng et al., 2006). Proteins are mainly adsorbed by the clays through electrostatic interactions and entropy gain. Adsorption of proteins and immobilization of enzymes by clays from various sources were found in many studies

(Bajpai and Sachdeva, 2002; Larsson and Siffert, 1983; Ralla et al., 2010; Sinegani et al., 2005). Therefore, the proteins were believed to be the major interfering compounds for reduced aflatoxin B₁ adsorption by smectites in fermentation solution, though some other organic compounds might have minor interferences.

V.4. Conclusions

The overall FTIR and XRD data confirmed that the interfering compounds were more potential than the aflatoxin molecules to occupy the interlayer exchange sites of the clays, which subsequently reduced aflatoxin adsorption by smectites.

Compared to the adsorption from simplified aqueous solutions, smectites had much lower aflatoxin B₁ adsorption capacities in corn fermentation solution. The FTIR and XRD investigations suggested that the protein substances from corn fermentation solution were the major interfering compounds, they occupied the interlayer space of smectites, and thus blocked the accessibility of the interlayer for aflatoxin B₁. The affinity of aflatoxin B₁ for the smectite's adsorption sites became weaker in the presence of such complex organic solution, as indicated by the reduced number of particular IR bands for aflatoxin B₁ adsorption. A considerably higher basal d₀₀₁ spacing of the smectite complexes in fermentation solution than pure smectites suggested that the interfering compounds had very strong affinity for the smectites.

However, despite of strong interferences of the proteins, the smectite's efficiency in adsorbing aflatoxin B₁ in fermentation solution could still be significant as smectites was evidenced to reduce aflatoxin toxicity in human and animal body in the presence of various biological components. Strategies should be developed to minimize the

interferences of the proteins or other compounds in fermentation solution for optimum aflatoxin adsorption by applying the worldwide recognized highly efficient bentonite clays.

CH APTER VI

MODIFYING SMECTITE WITH NUTRITIVE ORGANIC COMPOUNDS TO REDUCE PROTEIN'S INTERFERENCE ON AFLATOXIN B₁ ADSORPTION IN FERMENTATION SOLUTION

VI.1. Introduction

The carcinogenicity of aflatoxin B₁ due to consumption of mycotoxin contaminated foods is a serious concern for the agricultural, veterinary, and medical science researchers. Situation is the worst in developing countries with obligatory consumption of corn, limited facilities for mycotoxin control, and climatic conditions favorable for fungal growth.

Application of smectite minerals could be the most effective, safe, and economically feasible amelioration techniques for aflatoxin detoxification, and could save life from mycotoxin hazards. Smectites are able to remove mycotoxins, heavy metals, and other organic pollutants such as aromatic hydrocarbons and pesticides from various environments (Cruz-Guzman et al., 2004; Deng et al., 2003; Jaynes and Boyd, 1990; Laird et al., 1992; Lin, 2002; Lo et al., 1997).

Organo-smectites are the smectites whose interlayer surfaces are modified by treating with organic compounds. Surface modification of clay refers to the act of altering the physical, chemical or biological properties of the surface of clay. In various studies, clay minerals were modified by organic polyacids, non-ionic or cationic surfactants, and organic or inorganic cations.

Surfactant modified smectite showed greater aromatic chlorophenols adsorption from a pH 4.9 acetate buffer solution while unmodified smectite did not adsorb the molecules (Deng et al., 2003). Organically modified clays increased adsorption of various organic contaminants, and suggested the implication of those clays for many environmental remediation (Boyd et al., 1988; Jaynes and Boyd, 1990; Lee et al., 2004; Lo et al., 1997; Meier et al., 2001).

According to our previous studies, smectites had high aflatoxin adsorption capacity in aqueous, glucose, and ethanol solutions (Alam et al., 2015). The previous studies indicated that smectites had much lower aflatoxin B₁ adsorption in real fermentation solution. The overall findings from the previous chapter suggested that there might be some large biological molecules, most possibly protein in fermentation solution blocked most of the adsorption sites of smectites, and thus reduced their aflatoxin adsorption ability. To inhibit or limit the access of those interfering compounds but to achieve greater aflatoxin adsorption by the smectites in fermentation solution, modification of the smectites with small organic compounds might be an effective strategy.

In the present study six small, organic, and nutritive compounds were selected for surface modification of the smectite. The organic compounds were choline chloride (CC), L-carnitine hydrochloride (Car), L-arginine monohydrochloride (Arg), L-histidine monohydrochloride (His), L-lysine (Lys), and L-tryptophan (Trp). The choline and carnitine were the organic cationic compounds. The choline is an essential nutrient compound present in many foods. It is used as an essential dietary supplement,

especially in poultry feed. The carnitine is also known as food supplement nutrient compound, and is included under the vitamin B group. The arginine, histidine, lysine, and tryptophan (aromatic) were the basic amino acids. The amino acids are considered as the fundamentally essential components of life.

The sizes of six modifying compounds were smaller than the size of aflatoxin B₁. The molecular structures of the six organic compounds are shown in the Figure 39.

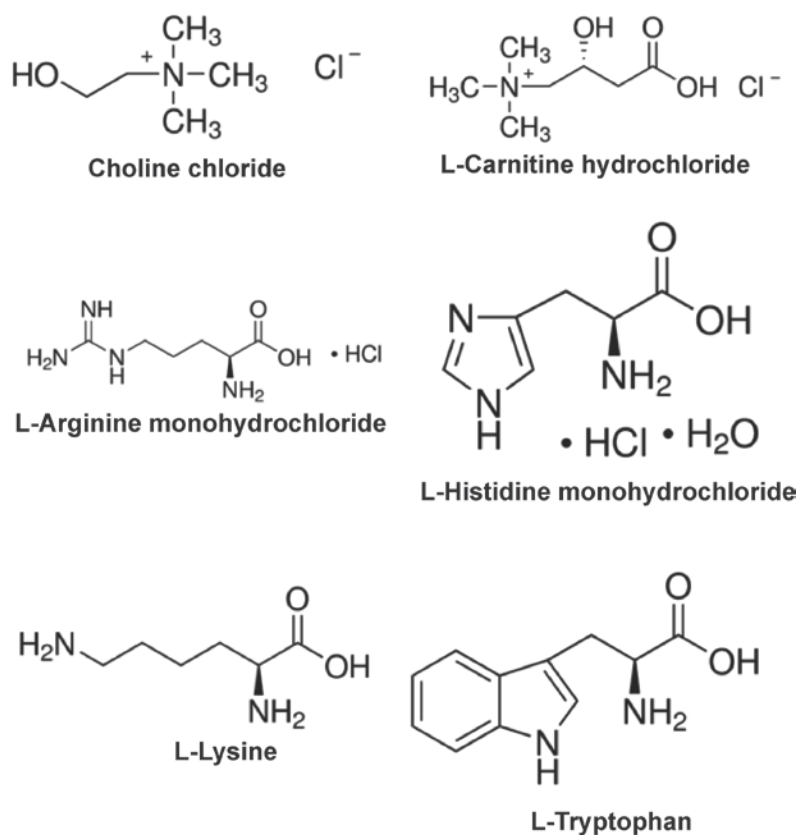


Figure 39. Chemical structures of the organic compounds used to modify the smectites.

The hypotheses, criteria, and logics on selecting the organic compounds and how they might work are described below:

VI.1.1. Primary adsorption of the organic cations and amino acids on clay

The primary adsorption of the organic cation in the smectite is a cation exchange phenomenon. The inorganic cations in the interlayer of smectite can easily be replaced by the organic cations of the modifying compounds. Both organic cations and aflatoxin molecules can be adsorbed in the interlayer of smectites as showed in the aflatoxin B₁-smectite model (Figure 40).

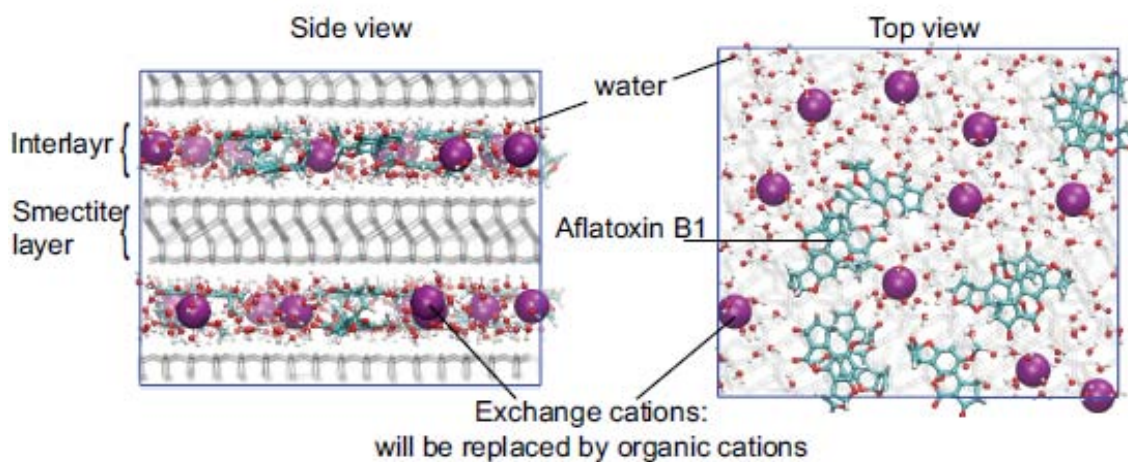


Figure 40. Model of AfB₁-smectite complex. The exchangeable cations in the interlayer of smectites can easily be replaced by organic cations. Adopted from Deng and Szczerba (2011).

Aflatoxin adsorption by smectite was increased or decreased by changing interlayer cations. The smectites saturated by divalent cations with low hydration radius such as Ba^{2+} showed higher aflatoxin adsorption than smectites treated by monovalent cations or divalent cations with high hydration energy (Deng et al., 2012). However, inorganic cations showed no effects on aflatoxin adsorption when tested in simulated gastric fluid containing pepsin i.e., protein (Barrientos-Velázquez et al., 2016).

The amino acids exerted different electric charges depending on the pH of solution, and their isoelectric point, PI (the pH at which a particular molecule carries no net electrical charge). The positively charged amino acids can behave like cations, thus can be adsorbed by the smectites. The molecular weight, pKa, and PI values of the compounds are presented in the Table 6. The pKa and PI values were not considered for choline and carnitine as they had positive charge in their structure.

Table 6. Some properties of the modifiers. Here, pKa_1 = α -carboxylic group, pKa_2 = α -amino group, pKa_3 = side chain group

| | Choline | Carnitine | Arginine | Histidine | Lysine | Trptophan |
|------------------------|----------------|------------------|-----------------|------------------|---------------|------------------|
| M.W. (g/mol) | 104.17 | 161.19 | 174 | 155.16 | 146.19 | 204.23 |
| pKa₁ | --- | --- | 2.17 | 1.82 | 2.18 | 2.83 |
| pKa₂ | --- | --- | 9.04 | 9.17 | 8.95 | 9.39 |
| pKa₃ | --- | --- | 12.48 | 6.00 | 10.53 | --- |
| PI | --- | --- | 10.76 | 7.59 | 9.74 | 5.89 |

VI.1.2. Secondary adsorption of the compounds on clay

The secondary adsorptions could be the hydrophobic interactions. The non-polar surfaces of the smectite's interlayer were believed to largely control aflatoxin adsorption. As the organic cations would decrease or expel the interlayer water content, it would increase the hydrophobicity of the clays' surface environment, which possibly would enhance aflatoxin adsorption. It is also not surprising that these amino acids can act like 'zwitterion', and attached to the non-polar surface of smectites.

Benzene and other aromatic hydrocarbon in water were adsorbed by the trimethylphenylammonium (TMPA)-smectite interlayer sites between the exchangeable cations because there were more non-polar adsorption sites (Jaynes and Boyd, 1991, 1990).

VI.1.3. Blocking of protein molecules but easy access of aflatoxin B₁

The purpose of smectite's modification was to block protein adsorption, and thus might indirectly enhance aflatoxin adsorption as there would be less competition among the large protein molecules and aflatoxin B₁ molecules for the clay's adsorption domains. The size of the small organic compounds would be such that they do not completely fill the interlayer spaces of the smectites. So, the interlayer adsorption sites of the clays would be large enough for the access of small aflatoxin B₁ but would be too small for the entrance of high molecular weight compounds like protein from fermentation solution. For example, the molar mass of calcium ion is 40.07 g mol⁻¹, whereas it is 104.17 g mol⁻¹ for cation choline. When the calcium ions in the interlayer

are exchanged by the choline, the spaces become limited for the proteins, yet large enough for aflatoxin.

Jaynes and Zartman (2011) showed that modification of clays with large organic cation such as hexadecyltrimethyl ammonium, HDTMA (m.w. 285 g mol^{-1}) completely filled the interlayer space of the clays, and hence largely reduced the aflatoxin adsorption. Therefore, molecular size of the modifiers was also very important criteria regarding aflatoxin adsorption. The compounds were also selected based on their structural functional groups that were supposed to be responsible for aflatoxin adsorption too.

VI.1.4. Practical reasons for selecting the compounds for modification

Commercially prepared organic clays might contain excess organic cations. The adsorbed organic cations and amino acids by the clays might partially be desorbed during the food digestion in animals. During biofuel production if the clay is added, it may end up in DDG. So, selecting the toxicity free compound for clay modification is crucial. Smectite modified with small organic compound such as TMPA (m.w. 136 g mol^{-1}) effectively adsorbed aflatoxins in the presence of soluble proteins (Jaynes and Zartman, 2011). The TMPA is toxic, and the regulatory authority e.g., FDA would not permit TMPA in animal or human food. On the contrary, the compounds used for modification in this study were essential nutrients required for proper physiological development in human and animal, and thus they would be accepted in foods and feeds.

Use of modified smectites for pollutants removal is not a new approach but no study was conducted evaluating the organo-smectite's aflatoxin B₁ adsorption in

fermentation solution during biofuel production. The main purpose of the proposed study was to reduce the protein's interference on aflatoxin B₁ adsorption by organically modified smectite in fermentation solution, so that higher aflatoxin adsorption can be achieved. Reaching to this goal had three specific objectives: 1) to observe smectite's intercalation of the organic compounds, 2) to investigate any effects of the intercalated cations and amino acids on the interlayer accessibility of protein and aflatoxin molecules by the XRD and FTIR, and 3) to observe the aflatoxin adsorption by the organo-smectites in fermentation solution and in simulated pure protein solution.

VI.2. Materials and Methods

VI.2.1. Selected smectites

The calcium smectite 3MS (NovasilTM 16) was selected for the study because of its high adsorption capacity ($Q_{\max} = 0.50 \text{ mol kg}^{-1}$ or 156 g kg^{-1}) and high affinity ($K_d = 1.02 \times 10^6 \text{ M}^{-1}$) for aflatoxin in simplified solution. The smectite 8TX and 6WY (bulk) were only used for a single preliminary experiment. The 8TX was native in Texas and 6WY was the sodium bentonite from Wyoming. Size fractionation and clay extraction procedures were described in detail elsewhere (Deng et al., 2012). The cation exchange capacity (CEC) of 3MS was measured as $94.0 \text{ cmol}^{(+)} \text{ kg}^{-1}$. The pH in water and electrical conductivity (EC) was recorded as 8.4 and $148 \mu\text{S cm}^{-1}$, respectively.

VI.2.2. Aflatoxin B₁ and corn fermentation solution

Information on collection of aflatoxin B₁ and fermentation solution, and their preparation for adsorption experiment was described thoroughly in the Chapter IV.

VI.2.3. Modification of smectite by choline, carnitine, arginine, histidine, lysine, and tryptophan

The six organic compounds with high purity (>98%) were purchased from Sigma Aldrich. The 1000 ppm stock solutions were prepared for choline chloride, carnitine, arginine, histidine, lysine, and tryptophan by dissolving the compounds in distilled water. The smectites were modified by these compounds following two kinds of treatments as described below:

Treatment 1 (T1) or light loading: About 100 mg of smectite was added to each of the eight 50 mL Nalgene plastic tubes (six tubes for 3MS and two tubes for 8TX and 6WY). Smectites were treated in such a way that the amounts of solution taken from each stock 1000 ppm solution was 2 times the CEC of the smectite to confirm adequate cation exchange. The clay suspensions in each tube were shaken on a rotary shaker at 200 rpm for 2 hours for cation exchange and adsorption. After shaking, the tubes were centrifuged at 2000 rpm and the clear supernatants were removed. The clay residues were washed off by distilled water for 2 times to remove the exchanged inorganic cations and excess organic compounds that were not adsorbed by the clay. About 5 mL of water was added to each tube and the stock organo-clay suspensions were kept in the refrigerator at 4 °C. The organo-smectites were designated by CC_{T1}-3MS, Car_{T1}-3MS, Arg_{T1}-3MS, His_{T1}-3MS, Lys_{T1}-3MS, Trp_{T1}-3MS, CC_{T1}-8TX, and CC_{T1}-6WY. The 8TX and 6WY was only modified with choline as a preliminary test to observe the effect of modification on adsorption of compounds from fermentation solution.

Treatment 2 (T2) or heavy loading: This time modification was done by treating the 3MS with respective solution containing the amounts of compounds that was 3 times the CEC of the clay, and the tubes were shaken for 24 hours. The treatment was repeated one more time, and finally the clay residues were washed off with water. This batch of organo-smectites was denoted by CC_{T2}-3MS, Car_{T2}-3MS, Arg_{T2}-3MS, His_{T2}-3MS, Lys_{T2}-3MS, and Trp_{T2}-3MS.

VI.2.4. Preparation of organo-smectite complexes for XRD and FTIR

To verify if the cations and amino acids were in the interlayer, and if they had any effect on reducing protein's adsorption by the smectites but maintaining the optimum aflatoxin B₁ adsorption, thermal XRD and FTIR investigations were performed for the organo-smectites. The organo-smectite complexes were added into clean fermentation solution, in artificially aflatoxin B₁ (8 ppm) spiked fermentation solution, and aflatoxin B₁ aqueous solution. The methodology followed for the FTIR and XRD analyses were described in detail by Alam et al. (2015). In this study for the thermal XRD analyses, the organo-smectites were heated at 200 °C to observe the intercalation of the compounds. To observe d₀₀₁ spacing after adsorption of compounds from fermentation solution and aflatoxin, smectite complexes were recorded at room temperature (23 °C) and heated in a furnace at 300 °C for 1 hour, and then immediately placed on the XRD holder for recording again.

VI.2.5. Procedures for aflatoxin B₁ adsorption isotherms by organo-smectites in corn fermentation solution, pure protein solution, and simulated fermentation solution (SFS)

The procedures for the analyses of aflatoxin adsorption in aqueous and fermentation solution were described by Alam et al. (2015), and in Chapter V, respectively.

In the current study, to observe the effects of modified smectites on aflatoxin adsorption in pure protein solution, adsorption experiment was conducted in different concentrations of pepsin solutions, where no other compounds were present to interfere the adsorption. To test aflatoxin adsorption in pepsin, single point (4 ppm AfB₁) adsorption experiment was performed using the smectites 3MS, CC_{T2}-3MS, and Car_{T2}-3MS. Pepsin (collected from porcine gastric mucosa) was purchased from Sigma-Aldrich. The 1000 ppm pepsin stock solution was prepared in distilled water. Then the solution was diluted to 10, 20, 50, 100 ppm. The 4 ppm aflatoxin B₁ solution was prepared in each pepsin solution. Three replications were considered for each clay sample. Two replications were taken for the blank. The same experiment was repeated one more time. In the first experiment, the adsorption was accomplished in pepsin solutions with their natural pH (6, 5.3, 4.7, 4.0), which was not controlled. In the second experiment, the pH was maintained at 6.0 for the all solutions. Before quantification of aflatoxin B₁ by the UV, absorbance of the pepsin solutions was checked (Figure 41). The proteins did not absorb UV at 365 nm. However, protein absorption was observed

between 200 and 250 nm wavelength, and increased with the protein concentration. The next procedures were same as mentioned earlier.

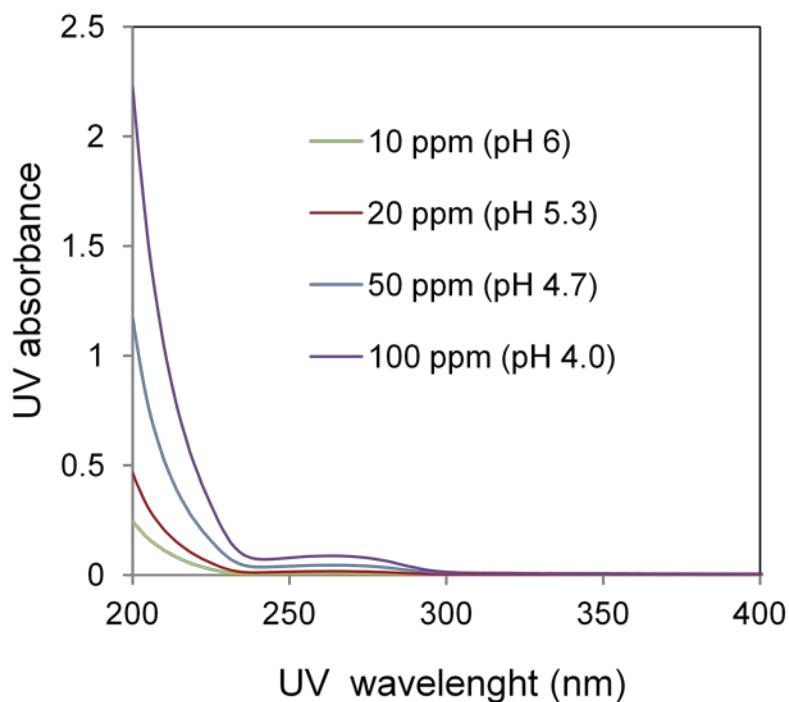


Figure 41. UV absorbance of protein in pepsin solution. The pH was measured at their natural conditions after dissolved in water.

Again, aflatoxin B₁ adsorption by the smectites was also tested in simulated fermentation solution (SFS), which was prepared by mixing 10% ethanol (v/v), 10% glucose (w/v), and 100 ppm pepsin in distilled water. The natural pH of SFS was 5.5. The procedures for isothermal adsorption were described earlier.

VI.2.6. Statistical analyses

One way Analysis of Variance (ANOVA) was conducted on the data to see if the organo-smectites significantly influenced the sorption of aflatoxin in protein solution or not. When there were significant differences ($p < 0.05$) among the treatments, Tukey's HSD was used to separate the means. Program JMP Pro 12 was used in the statistical analyses.

VI.3. Results and discussions

VI.3.1. XRD analyses of smectite and choline-smectites in aqueous and fermentation solution

To verify if the organic cations were in the interlayer of smectites, and to observe if they had any impact on reducing protein adsorption but allowed optimum aflatoxin adsorption, choline was firstly tested to modify the smectites. The d_{001} spacing of the choline modified 3MS, 8TX, and 6WY after being treated in three different solutions (aflatoxin B₁ in water, fermentation solution, and aflatoxin B₁ spiked fermentation solution) was recorded at 23 °C and after heating at 300 °C (Figure 42).

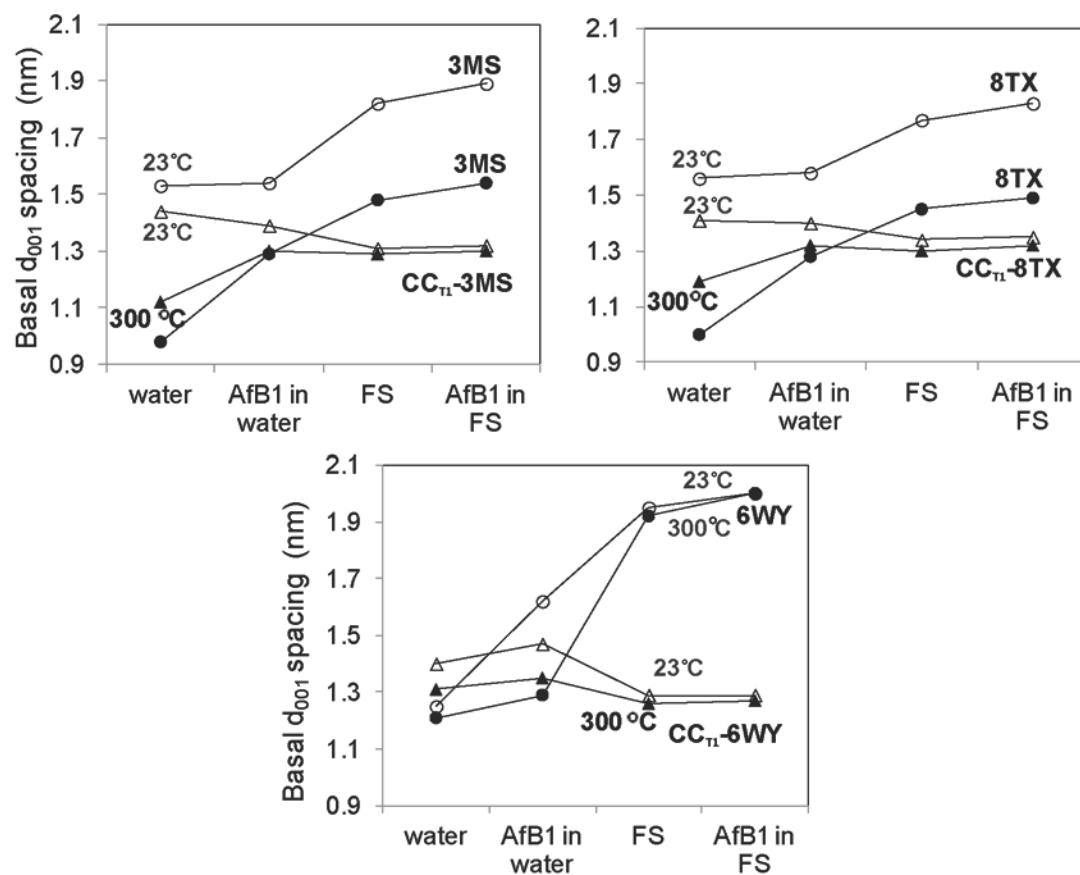


Figure 42. The d_{001} spacing of three natural smectites and respective choline-smectites, and their complexes after reacting in three different solutions recorded at room temperature and 300 °C.

VI.3.1.1. Presence of choline in interlayer of smectites

The presence of choline in the smectites was confirmed by the XRD analysis. At room temperature, basal d_{001} spacing of CC_{T1}-3MS and CC_{T1}-8TX dispersed in water was lower than the spacing of unmodified 3MS and 8TX, respectively. After heating at 300 °C, the d_{001} spacing of 3MS and 8TX was collapsed to 0.98 nm and 1.0 nm, respectively but the CC_{T1}-3MS and CC_{T1}-8TX expanded to 1.12 nm and 1.19 nm, which indicated the organic cations were in the interlayer. The 6WY did not collapsed to 1.0 nm after heating, most probably due to its expansion for rapid water absorption from the atmosphere. However, choline was also in the interlayer of 6WY.

VI.3.1.2. Access of aflatoxin B₁ in organo-smectites

When the smectite complexes were in the aflatoxin B₁ solution prepared in water, at room temperature the cations decreased the water content of the smectites but the d_{001} spacing were similar (~1.30 nm) after heating for both the 3MS and organo-smectites. This suggested that the aflatoxin B₁ was accessible to the organo-smectites, otherwise the d_{001} spacing would have reduced to ~1.0 nm. The d_{001} spacing of the CC_{T1}-6WY increased slightly after saturating in aflatoxin, suggesting less aflatoxin B₁ intercalation by the 6WY.

VI.3.1.3. Reduced adsorption of interfering compounds by organo-smectites in fermentation solutions (no AfB₁) and AfB₁ spiked fermentation solutions

Encouraging results were obtained when the organo-smectites were treated in fermentation solutions. Both at low and high temperature, the d_{001} spacing of CC_{T1}-3MS,

CC_{T1}-8TX and CC_{T1}-6WY were reduced greatly compared to their respective pure smectite. At room temperature, d_{001} spacing was reduced from 1.82 to 1.31 nm; 1.77 to 1.34 nm; and 1.95 to 1.29 nm for CC_{T1}-3MS, CC_{T1}-8TX, and CC_{T1}-6WY, respectively. In this case, the basal spacing reduction might be partially attributed to some water expulsion from the interlayer of smectites. Again, after heating at 300 °C, d_{001} spacing for same smectite complexes were reduced from 1.47 to 1.29 nm; 1.49 to 1.30 nm; and 1.92 to 1.26 nm. Similar observations were obtained when the smectites reacted in fermentation solution containing aflatoxin B₁. Basal spacing was also reduced for the organo-smectites. The d_{001} spacing was reduced from 1.89 to 1.32 nm; 1.83 to 1.35 nm; and 2.0 to 1.29 nm for CC_{T1}-3MS, CC_{T1}-8TX, and CC_{T1}-6WY, respectively at room temperature. After heating, d_{001} spacing for the same smectite complexes was minimized from 1.54 to 1.30 nm; 1.49 to 1.32 nm; and 2.0 to 1.27 nm. The higher basal spacing of 6WY even after heating suggested that this clay might be more accessible to those compounds than the calcium smectites. This investigation confirmed that choline intercalation of the smectites prohibited at least some adsorption of the interfering compounds from going into the interlayer of smectites when they were exposed to fermentation solution.

VI.3.2. Smectite's intercalation of organic cation and amino acids revealed by FTIR and XRD

Only 3MS was selected for further modification with other organic compounds. The 6WY might be suitable for rapid cation exchange but it was not preferred as sodium bentonite was known to have low aflatoxin adsorption capacity. Moreover, a study showed that sodium saturated smectite had very higher pepsin (protein) adsorption than the calcium smectite (Barrientos-Velázquez et al., 2016). The higher d_{001} spacing of 6WY compared to the calcium smectites after reacting in fermentation solution illustrated in Figure 42 was in agreement with that investigation.

The FTIR results (Figure 43) confirmed that the smectite's interlayer was intercalated with choline and carnitine after the first or light treatment (T1). An intense treatment was required for arginine and histidine to saturate the smectite. Negligible or no band appeared even after intensive treatment (T2) of smectite with lysine and tryptophan.

There was no difference in the band position between the spectra of CC_{T1}-3MS and CC_{T2}-3MS, or between Car_{T1}-3MS and Car_{T2}-3MS. The common bands evolved due to intercalation of choline and carnitine occurred at 1474 and 1420 cm^{-1} . Bands at 1717, 1487, 1455, 1401, and 1362 cm^{-1} appeared for carnitine intercalation. Remarkable changes occurred in the spectra of Arg_{T2}-3MS and His_{T2}-3MS when the smectite was intensively treated with arginine and histidine. The amino acids intercalation bands were observed at 1673, 1475, 1455, 1401, 1345 cm^{-1} (Arg_{T2}-3MS); 1724, 1506, 1405 cm^{-1} (His_{T2}-3MS).

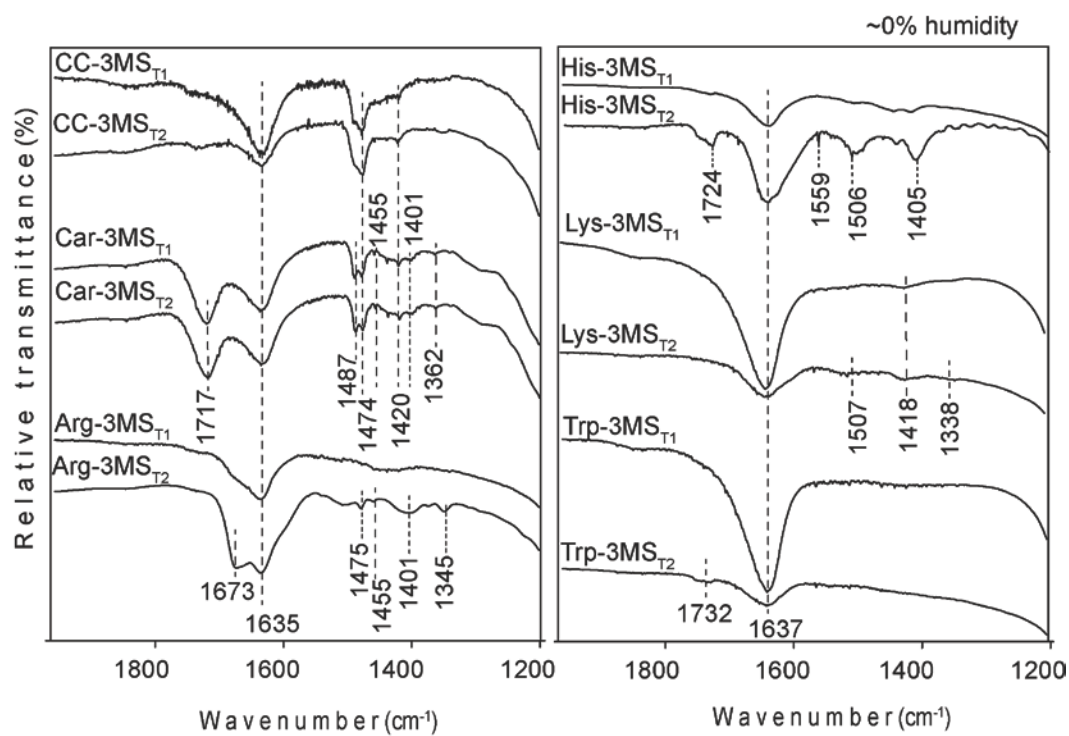


Figure 43. Intercalation of organic cations and amino acids revealed by FTIR.

The basal spacing of the smectite and organo-smectites recorded by thermal XRD analyses is presented in the Table 7.

The d_{001} spacing of organo-smectites (except Trp_{T1}-3MS, $d_{001} = 1.53$ nm, and Lys_{T1}-3MS, $d_{001} = 1.52$ nm) were less than the basal spacing of 3MS ($d_{001} = 1.53$ nm) at room temperature, indicating the removal of internal water molecules from the smectite. Earlier study concluded that water adsorption was greatly declined by the clays treated with some compounds consisting large organic cations (Grim et al., 1947). After heating at 200 °C no organo-smectite was collapsed to 0.98 nm. The minimum d_{001} spacing was 1.03/1.03 nm for Trp_{T1}-3MS or Lys_{T1}-3MS, and maximum 1.28 nm for Arg_{T2}-3MS. The smectite was intercalated with carnitine (1.16 nm) and choline (1.12 nm) even after light loading treatment, whereas the other compounds were not. These cations easily exchanged the inorganic cations. Though the d_{001} increased up to 1.19 for lysine, the bands were very weak on Lys_{T2}-3MS spectra, showed in the Figure 43.

The d_{001} increased for the Car_{T2}-3MS than the Car_{T1}-3MS but no additional band appeared in the IR spectra upon intense treatment, which was in agreement with study by Cruz-Guzman et al. (2004). The overall XRD data indicated that the organic cations and amino acids were in the interlayer of smectites in varying degrees. The CC_{T1}-3MS was not expanded anymore after second treatment, suggesting the smectite was fully saturated with choline after first time loading.

Table 7. Basal d_{001} spacing (nm) of smectite and organo-smectites analyzed by XRD.

| Smectite | 23 °C | 200 °C |
|------------------------|-------|--------|
| 3MS | 1.53 | 0.98 |
| Car _{T1} -3MS | 1.41 | 1.16 |
| Car _{T2} -3MS | 1.37 | 1.23 |
| CC _{T1} -3MS | 1.44 | 1.12 |
| CC _{T2} -3MS | 1.42 | 1.12 |
| His _{T1} -3MS | 1.34 | 1.08 |
| His _{T2} -3MS | 1.29 | 1.19 |
| Lys _{T1} -3MS | 1.52 | 1.03 |
| Lys _{T2} -3MS | 1.42 | 1.19 |
| Arg _{T1} -3MS | 1.35 | 1.04 |
| Arg _{T2} -3MS | 1.33 | 1.28 |
| Trp _{T1} -3MS | 1.53 | 1.06 |
| Trp _{T2} -3MS | 1.46 | 1.03 |

VI.3.3. XRD analyses of organo-smectites in aqueous and fermentation solution

Encouraged from the observation of choline-smectite to reduce protein adsorption from fermentation solution, similar experiments were carried out for other five organo-smectites. The organo-smectites were analyzed after reaction in fermentation solution, aflatoxin B₁ in water, and aflatoxin B₁ in fermentation solution by XRD to observe any differences in their d_{001} spacing with unmodified smectite.

VI.3.3.1. The d_{001} spacing of organo-smectites after treated in aflatoxin B₁ spiked aqueous solution

The basal spacing of the organo-smectites at room temperature was arbitrary to explain. The remarkably lower basal spacing of His_{T1}-3MS at this condition was possibly due to greater water removal from interlayer by histidine. After heating at 300 °C, all smectites showed similar d-values (1.27 to 1.29 nm), indicating that aflatoxin B₁

molecules were in the interlayer of organo-smectites. At least majority of aflatoxin's interlayer adsorption was confirmed in the organo-smectites, when it was observed that both Car_{T1}-3MS (well-intercalated) and Trp_{T1}-3MS (almost no intercalated) had the same d₀₀₁ spacing. If the Car_{T1}-3MS was not saturated with aflatoxin B₁, the basal spacing would decline to ~1.16 nm after heating (Figure 44).

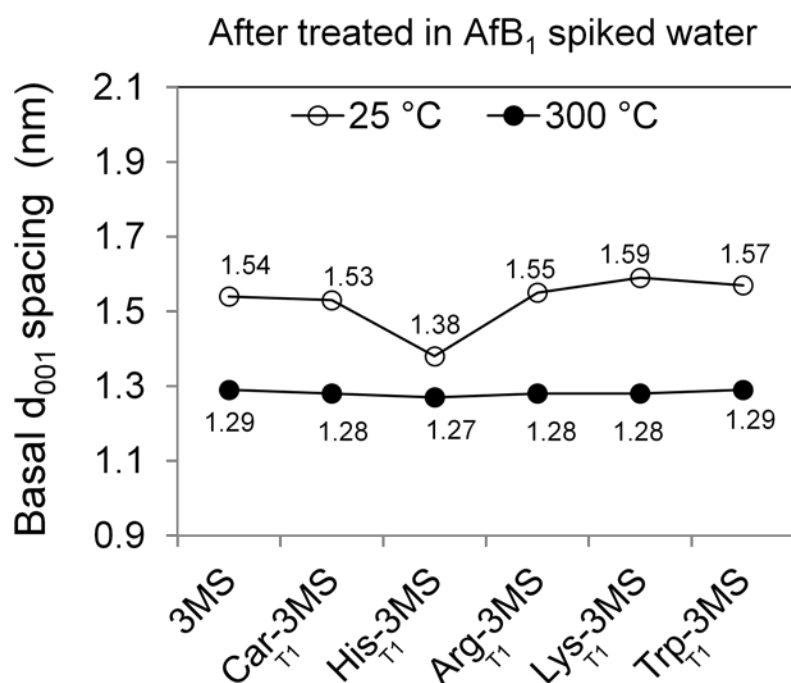


Figure 44. The d₀₀₁ spacing of smectite and its complexes after treated in AfB₁ spiked water.

VI.3.3.2. The d_{001} spacing of organo-smectites after treated in fermentation solution

At room temperature, the d_{001} spacing reduced for Car_{T1}-3MS (1.38 nm), His_{T1}-3MS (1.66 nm), His_{T2}-3MS (1.32 nm), Arg_{T1}-3MS (1.75 nm), Arg_{T2}-3MS (1.39 nm), and Lys_{T1}-3MS (1.77 nm) compared to the 3MS (1.82 nm), suggesting water expulsion as well as reduced adsorption of interfering compounds from the fermentation solution. After heating at 300 °C, the d_{001} spacing of all organo-smectites reduced to varying degrees compared to the 3MS (1.48 nm). The basal spacing was reduced most for the Car_{T1}-3MS (1.21 nm). Greater basal spacing reduction was observed for the His_{T2}-3MS & Arg_{T2}-3MS than the His_{T1}-3MS, & Arg_{T1}-3MS because of greater intercalation of the amino acids after second treatment (Figure 45).

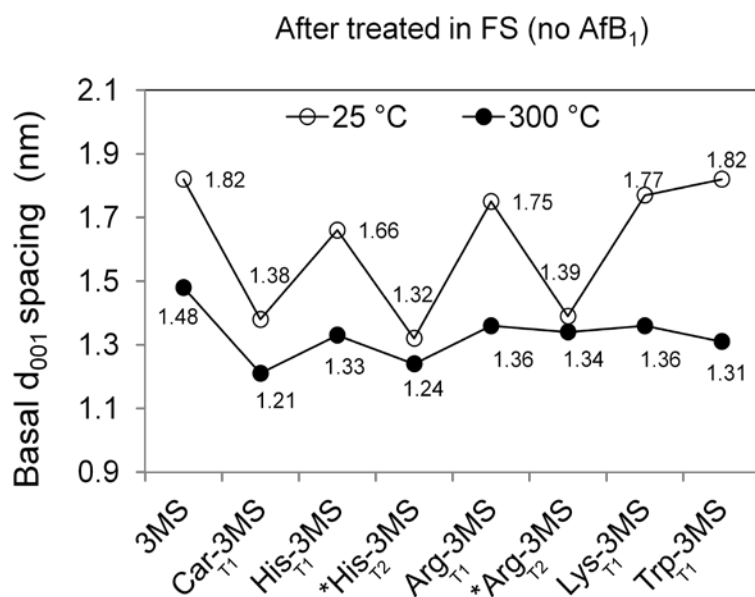


Figure 45. The d_{001} spacing of smectite and its complexes after treated in fermentation solution.* indicated organo-smectites given T2.

VI.3.3.3. The d_{001} spacing of organo-smectites after treated in aflatoxin B₁ spiked fermentation solution

After heating at 300 °C, the d_{001} spacing was reduced for the smectites at varying degrees (1.89 to 1.37 nm) while exposed to solution containing both aflatoxin B₁ and other biological molecules from fermentation solution. After heating, d_{001} spacing of 3MS was recorded at 1.54 nm, whereas for Car_{T1}-3MS, His_{T1}-3MS, Arg_{T1}-3MS, Lys_{T1}-3MS, and Trp_{T1}-3MS the values were 1.29, 1.36, 1.39, 1.4, and 1.39 nm, respectively. Therefore, both at room and elevated temperature, the lowest d-value was observed for Car_{T1}-3MS. Again, the remarkable reduction could be attributed to water expulsion and protein inhibition (Figure 46).

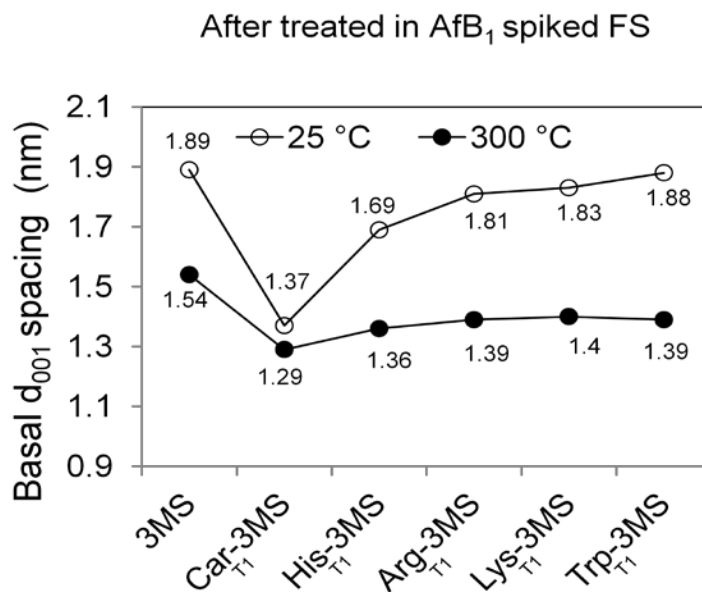


Figure 46. The d_{001} spacing of smectite and its complexes after treated in AfB₁ spiked fermentation solution.

The overall thermal XRD experiments in this study suggested that the choline and carnitine were the best of the selected compounds for surface modification of smectite because their intercalation in the smectite was easy and faster than the amino acids. When the organic modifiers were well intercalated they restricted the access of some of the interfering compounds but still allowed aflatoxin adsorption. After choline and carnitine, arginine and histidine could be the next two good choices to enhance smectite's aflatoxin adsorption in fermentation solution.

VI.3.4. FTIR analyses of organo-smectites in aqueous and fermentation solution

The FTIR spectra of smectite and two organo-smectites after adsorption of aflatoxin B₁ in aqueous solution (upper three spectra) and in fermentation solution (lower three spectra) showed almost similar aflatoxin B₁ band intensities and positions among the 3MS-AfB₁, CC_{T1}-3MS-AfB₁, and Car_{T1}-3MS-AfB₁ complexes (AfB₁ was prepared in water) (Figure 47). Only few bands were distorted or absent in the modified smectites (1363, 1482, and 1505 cm⁻¹). No distinguished differences were observed among the 3MS-AfB₁-FS, CC_{T1}-3MS-AfB₁-FS, and Car_{T1}-3MS-AfB₁-FS complexes. The protein bands were present in the organo-smectites, indicated that some protein compounds were still in the interlayer of those smectites. Few aflatoxin B₁ bands were also observed on those spectra.

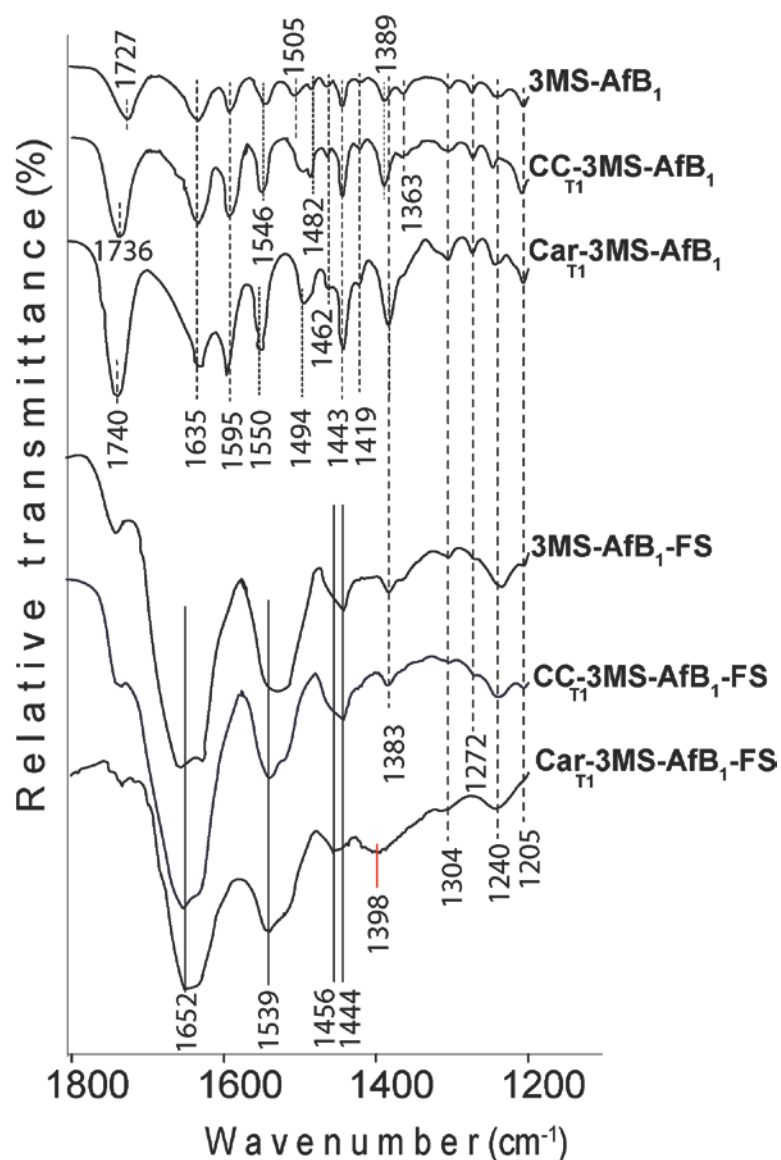


Figure 47. IR spectra of the three smectite-AfB₁ complexes after dispersing the smectites in AfB₁ spiked water (upper three) and AfB₁ spiked fermentation solution (lower three). The solid lines indicated bands for protein. The dotted lines were the bands for AfB₁, and red band 1398 cm⁻¹ could be due to carnitine. The spectra of 3MS-AfB₁ was recorded at 0% humidity, all others were recorded at room humidity (~24%).

As the IR bands confirmed the presence of the two organic cations (choline and carnitine) and two amino acids (arginine and histidine) in smectites, the four organo-smectites after reacting in fermentation solutions were investigated by FTIR to observe any effects of them on protein inhibition into the smectites (Figure 48).

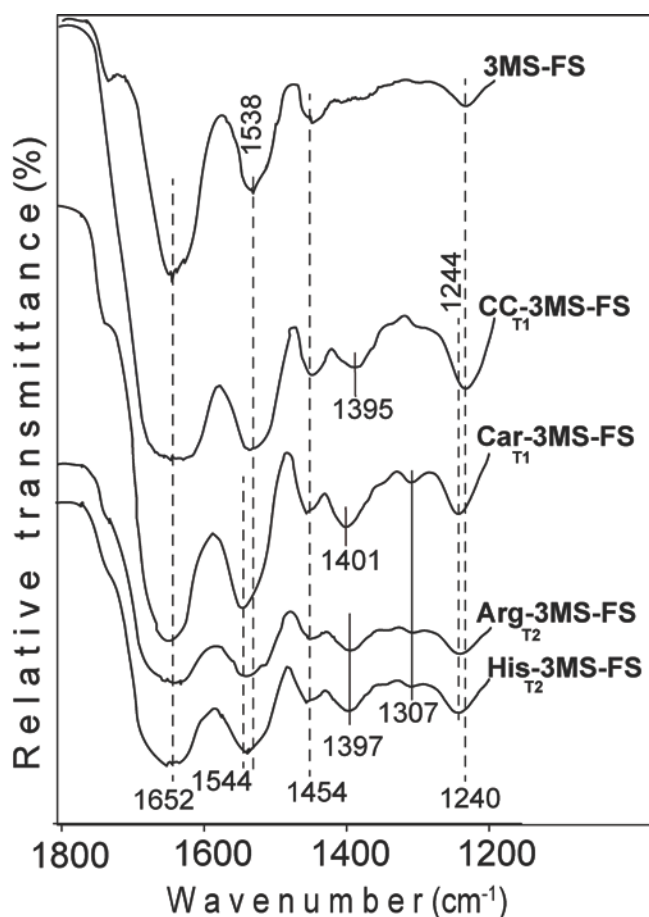


Figure 48. IR spectra of clay residues after reacting in fermentation solution. The spectra were recorded at room humidity (~25%). The dotted lines were for the protein bands. The solid lines were probably due to organic cations and amino acids intercalation.

Results showed that the IR bands of protein molecules such as ~ 1652 , 1538 , 1454 , 1240 cm^{-1} were still on the organo-smectites. The intensity of bands 1652 cm^{-1} and 1544 cm^{-1} became lower in Arg_{T2}-3MS-FS and His_{T2}-3MS-FS compared to other spectra. The lower intensity of the band 1652 cm^{-1} could be due to the water expulsion from the interlayer of smectite, as this band overlapped the water absorption band. For verification that these bands were not from the residue of fermentation solution (probably some residues could be mixed with the clay sample), the spectra for the residue of fermentation solution was subtracted from the spectra of clay complex. But still the major protein bands were present. Other bands at region 1307 , 1395 , 1397 , and 1401 cm^{-1} were most possibly from the intercalation of the amino acids on the smectites. The overall FTIR data suggested that though the organic cations could block some proteins as revealed by the XRD, but still a great portion of these molecules could access into the smectites. This investigation again reflected strong affinity of the protein molecules to the smectites.

VI.3.5. Aflatoxin B₁ adsorption by organo-smectites in aqueous and fermentation solution

Aflatoxin B₁ adsorption isotherms in aqueous and fermentation solution were performed for the unmodified and organo-smectites. It was revealed from the adsorption isotherm experiment that at temperature 25 °C and pH 6.0, aflatoxin adsorption by the two organo-smectites (Carnitine and choline modified) was quite high in 100% aqueous solution. The Q_{\max} values calculated by using Exponential Langmuir equation (ELM) were 0.55 and 0.51 mol/kg for Car-3MS and CC-3MS, respectively (Figure 49).

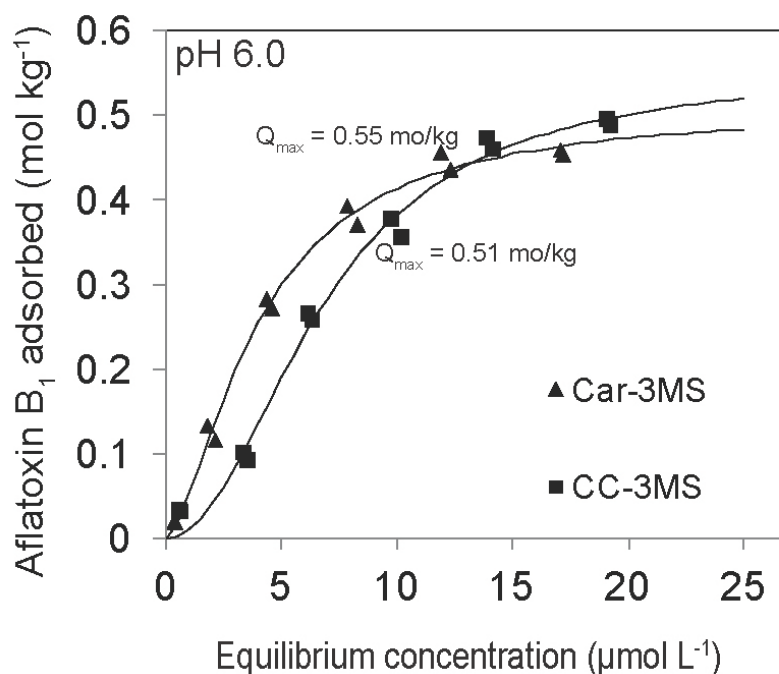


Figure 49. Aflatoxin B₁ adsorption isotherms of choline and carnitine modified smectites in 100% aqueous solution.

Aflatoxin B₁ adsorption isotherms (data were best fitted to the modified Langmuir, QKLM) of the original smectite and four organo-smectites CC_{T2}-3MS, Car_{T2}-3MS, Arg_{T2}-3MS, and His_{T2}-3MS in diluted fermentation solution (1:3) revealed that adsorption increased for the all organo-smectites to some extent compared to the adsorption by the same smectite before modification (Figure 50).

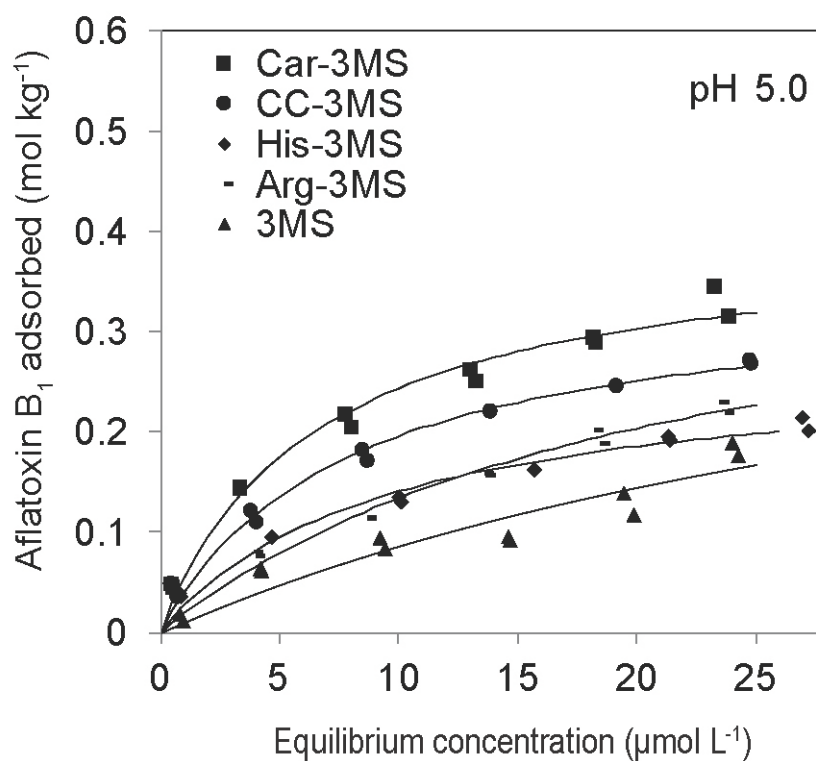


Figure 50. Aflatoxin adsorption isotherms of 3MS and four organo-smectites in fermentation solution.

The lowest adsorption was found for the 3MS (0.22 mol kg⁻¹) and the highest adsorption was observed for the Car_{T2}-3MS (0.45 mol kg⁻¹), indicating that adsorption increased almost double for that organo-clay (Table 8). Among the organo-smectites, lowest adsorption was exhibited by the His_{T2}-3MS e.g., 0.33 mol kg⁻¹. However, aflatoxin adsorption by the choline (0.44 mol kg⁻¹), and carnitine (0.45 mol kg⁻¹) modified smectites in fermentation solution was still below than the adsorption of the 3MS (0.50 mol/kg) in aqueous solution.

Table 8. AfB₁ adsorption fit parameters (QKLM) for organo-smectites in FS

| Media | Q _{max} (mol kg ⁻¹) | K _d (μM ⁻¹) | b | η ² |
|------------------------|--|------------------------------------|-------|----------------|
| 3MS | 0.22 | 0.031 | -3.89 | 0.92 |
| Car _{T2} -3MS | 0.45 | 0.165 | 0.78 | 0.97 |
| CC _{T2} -3MS | 0.44 | 0.12 | 1.24 | 0.98 |
| Arg _{T2} -3MS | 0.38 | 0.048 | -0.40 | 0.97 |
| His _{T2} -3MS | 0.33 | 0.105 | 1.45 | 0.97 |

VI.3.6. Aflatoxin B₁ adsorption by organo-smectites in pepsin solutions

According to the single point adsorption experiment, the aflatoxin B₁ adsorption in pure protein solution was significantly increased for the organo-smectites, CC_{T2}-3MS and Car_{T2}-3MS compared to the 3MS irrespective of the concentration of pepsin (Figure 51). In general, aflatoxin adsorption decreased with increased concentration of pepsin for all smectites. However, there might be some influences of pH on the adsorption as the pepsin solutions had different pH values, and the drastic fall of adsorption by 3MS at highest pepsin concentration could be due to low pH.

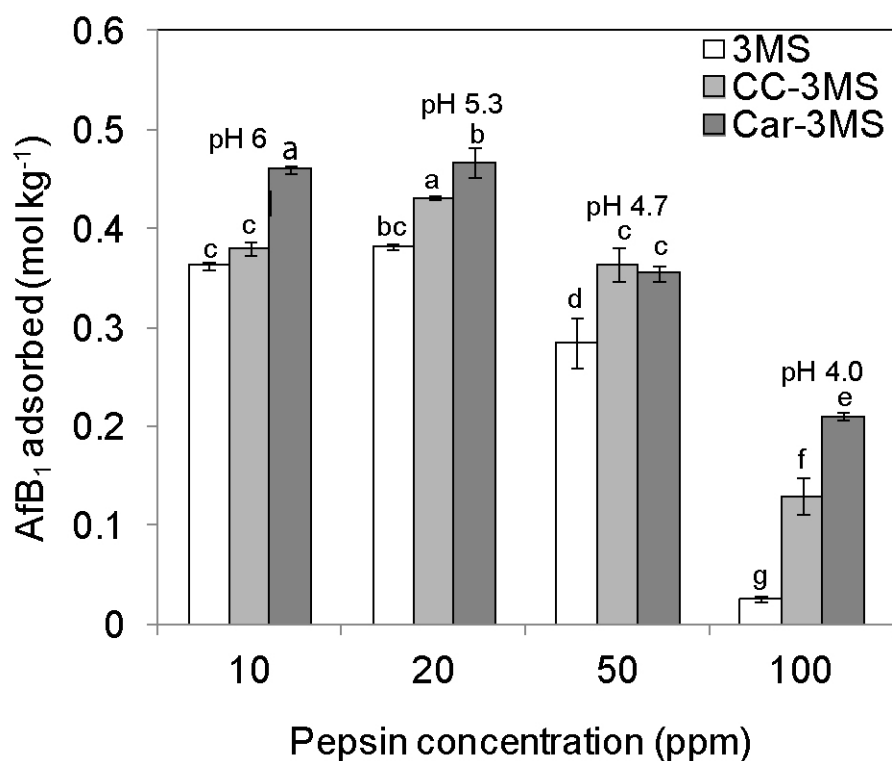


Figure 51. AfB₁ adsorption by the smectites in different concentration of pepsin solutions. The pH of the solutions was kept at their natural condition. The temperature was 25 °C. Means with the same letter were not significantly different. The significant levels (Tukey's HSD, $p < 0.05$) were compared among the three smectites for each concentration.

The adsorption experiment was repeated using the two highest concentrations of pepsin solution, and in distilled water under same pH treatment. Aflatoxin adsorption increased significantly ($p < 0.05$) at each point for the organo-smectites (Figure 52).

Increased aflatoxin adsorption in aqueous solution by the organo-clays is not unusual. An Wyoming montmorillonite, modified with three natural organic cations especially, the carnitine enhanced the adsorption of a herbicide simazine in aqueous solution (Cruz-Guzman et al., 2004). It was confirmed from this study that whatever the protein concentration and pH of the solution, the organo-smectite increased the aflatoxin

adsorption. Statistical analyses revealed that there was significant (<0.05) negative correlation between the protein concentration and aflatoxin adsorption (attached in Appendix).

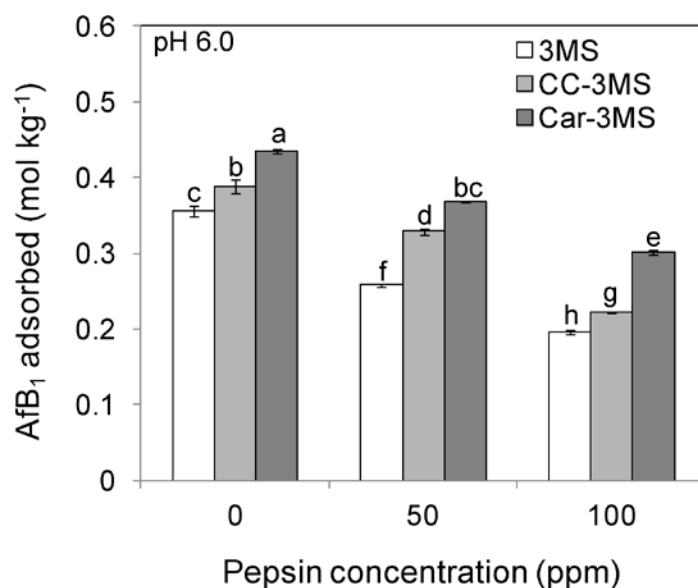


Figure 52. AfB₁ adsorption by the smectites in pepsin solutions at pH.6.0, and 23 °C. Means with the same letter were not significantly different. The significant levels (Tukey's HSD, $p = <0.05$) were compared among the three smectites for each concentration as well as among different concentrations because treatments were non-biased.

VI.3.7. Aflatoxin B₁ adsorption by organo-smectites in simulated fermentation solution (SFS)

Aflatoxin B₁ adsorption was significantly ($p = <0.0001$) low in SFS (10% ethanol, 10% glucose, and 100 ppm pepsin) than the adsorption in 100% aqueous solution. Encouragingly, carnitine-smectite also significantly ($p = <0.001$) increased the adsorption in SFS at any level of aflatoxin concentrations (except at 8 ppm) but yet still below the adsorption in aqueous solution (Figure 53). This study suggested that possibly the protein molecules reduced aflatoxin adsorption in SFS, as in the previous study it was found that the adsorption in 20% ethanol solution was still much high (Figure 23).

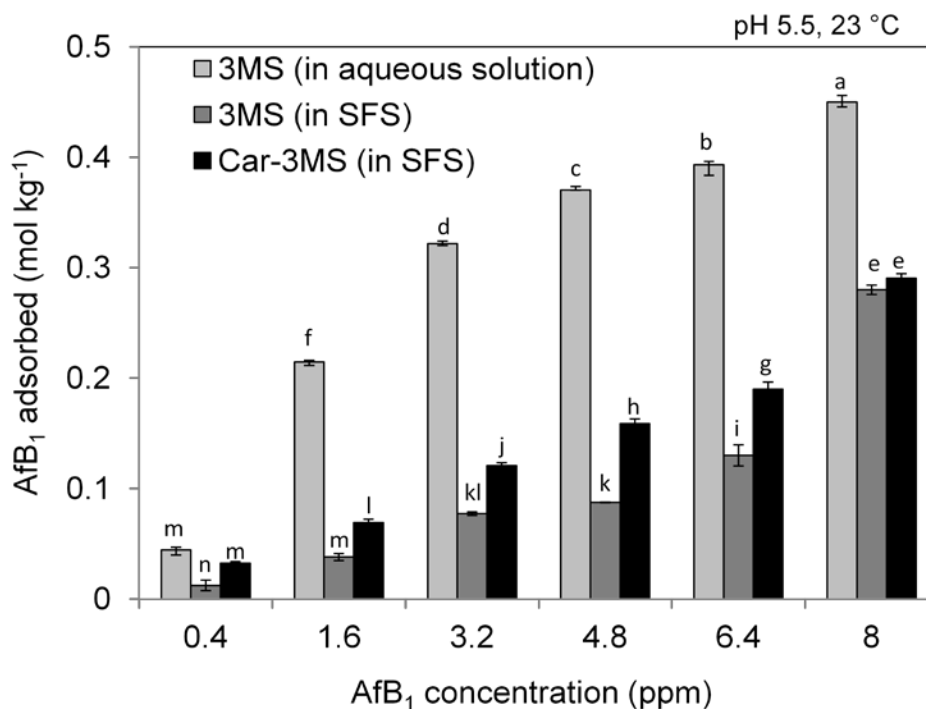


Figure 53. AfB₁ adsorption by the 3MS in aqueous and SFS (10% ethanol, 10% glucose, and 100 ppm pepsin), and effects of Car-3MS on AfB₁ adsorption in SFS.

This study revealed that it required less time and also less quantity of materials to get the clays well saturated with choline and carnitine than the amino acids, and it was probably because of the positive charges in the structure of the cationic compounds. The tryptophan was found to be bonded with smectite very weakly even after high loading treatment as indicated by the FTIR and XRD analyses. This might be due to its low isoelectric point (PI) value. Tryptophan was expected to have no effects on aflatoxin adsorption ability by the sodium smectite in the gastrointestinal tract of birds, and suggested to have reversible weak binding to the smectites (Magnoli et al., 2014). However, another study suggested tryptophan molecules having kinetic diameter of 0.6 nm easily entered in to the interlayer space of montmorillonite (Titus et al., 2003). Lower intensive IR bands of lysine on smectite suggested that the clays were not well saturated with this amino acid. Perhaps, even higher concentrated lysine would be needed for better lysine intercalation. Researchers investigated the lysine adsorption using concentration from 0.025 to 0.4 M, where they showed that at 0.4 M concentration lysine replaced only one third of the original interlayer cations of the smectite (Parbhakar et al., 2007). Their chemical and FTIR analyses revealed that lysine adsorption was primarily dominated by cation exchange followed by adsorption of electrically neutral lysine as zwitterion.

The important finding from the previous and also of the present study suggested that almost irreversibly bonded proteins on clay could be the main constraints for aflatoxin adsorption in fermentation solution. Research indicated that a high percentage (89%) of the total protein adsorbed to the mineral surfaces was still bounded to the

surfaces of protein-mineral (birnessite) complex even after thorough water washing (Naidja et al., 2002). However, in the case of birnessite the proteins were not intercalated in the mineral oxide but immobilized at the external surfaces and edges.

VI.4. Conclusions

Surface modification of smectite by small, nutritive organic cations, and amino acids reduced at least partially the protein adsorption, and inversely increased aflatoxin B₁ adsorption in fermentation solution as revealed by the XRD and isotherm adsorption experiments. The increased aflatoxin adsorption in fermentation solution could be due to blocking protein's access in the interlayer of smectites, and therefore more adsorption sites for aflatoxin molecules to occupy. The advantageous role of organo-smectites in aflatoxin adsorption was confirmed when they increased the adsorption in pure protein solution, and simulated fermentation solution treated under same pH conditions. The carnitine modified smectite was considered as the best aflatoxin B₁ adsorbents among the organo-smectites. However, the adsorption capacity and affinity was still lower than the adsorption in aqueous solution. In conclusion, proteins in fermentation solution were suggested to be the major interfering components for aflatoxin adsorption. Therefore, complete prohibition of the protein intercalation might not be possible. The presented investigation could be an example of minimizing the protein interference, and of maximizing aflatoxin sequestration by incorporating nutritive organic smectites into the fermentation system during biofuel production.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Aflatoxins are carcinogenic mycotoxins responsible for aflatoxicosis in animals and humans. Aflatoxins can be fatal if ingested at high concentration with foods. Long term research on aflatoxins suggested that they could be the direct or indirect reason for many health problems. Despite of taking many preventive measures to eradicate aflatoxins, their occurrence was found to be inextricable.

In public's view, it seemed to be rational to use the mycotoxin contaminated corn for biofuel production as it would reduce the burden of contaminated corn in the market and would save the public health from severe toxicity. The manifestation of three-fold increment of aflatoxin concentration in the co-product of biofuel, the popular animal feed, has attained the attention on how the toxicity could be reduced or diminished during ethanol production.

In this study some smectites were incorporated in simulated and real corn fermentation solutions to observe their efficiency in removing aflatoxins. The smectites were highly effective in binding aflatoxin in aqueous, ethanol, and glucose solutions but were much less efficient in binding aflatoxin in real fermentation solution. The ethanol and glucose, two of the major compounds in fermentation solution, had minimal interferences on aflatoxin adsorption. This study suggested that large protein compounds in fermentation solution were responsible for lower aflatoxin adsorption. The protein molecules blocked the potential adsorption sites of smectites for aflatoxins.

Surface modification of smectite by small, nutritive, cationic, and amino acid organic compounds reduced protein interlayer access to some extent, and thereby increased aflatoxin adsorption. The carnitine modified smectite performed the best in increasing aflatoxin adsorption in fermentation solution. The choline and carnitine modified smectites were also evaluated for their aflatoxin adsorption in pure protein solution. These organo-smectites significantly increased the aflatoxin adsorption compared to the adsorption by the unmodified smectite in protein solution. Significant negative relationship was observed between the aflatoxin adsorption and protein concentration, which confirmed that protein could block the clay's adsorption sites for aflatoxin.

In conclusion, the organo-smectites, especially the carnitine-smectite could be used effectively in the fermentation system to reduce the bioavailability and toxicity of aflatoxins that would otherwise concentrated in the dried distiller's grains. This amelioration technique to reduce aflatoxin would be economically feasible due to low cost of both smectites and the organic cations, and would be safe to human and animal health.

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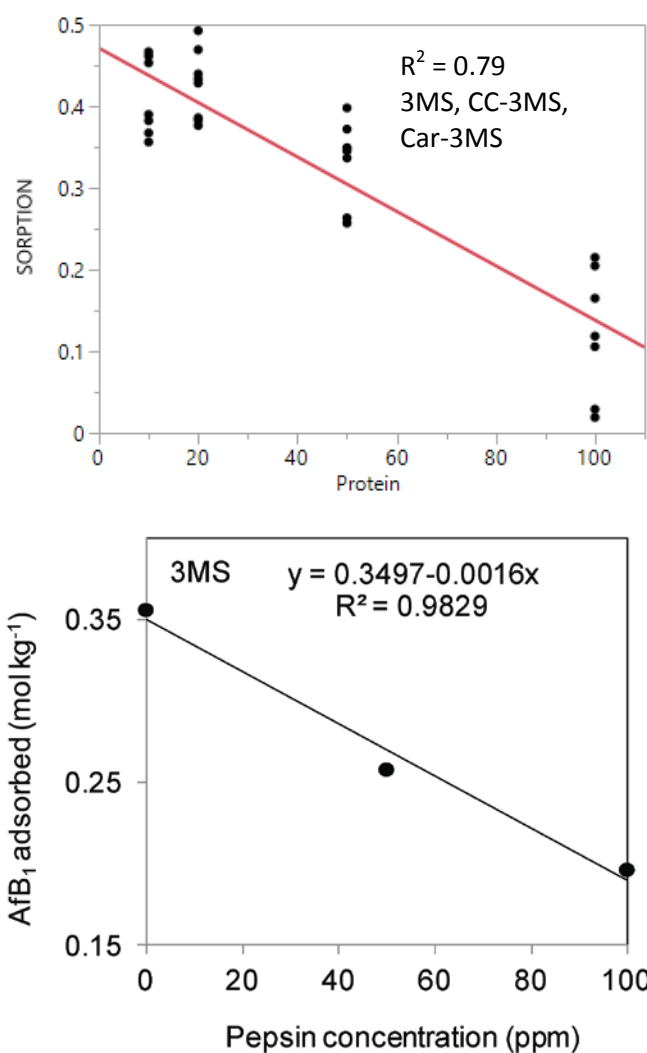
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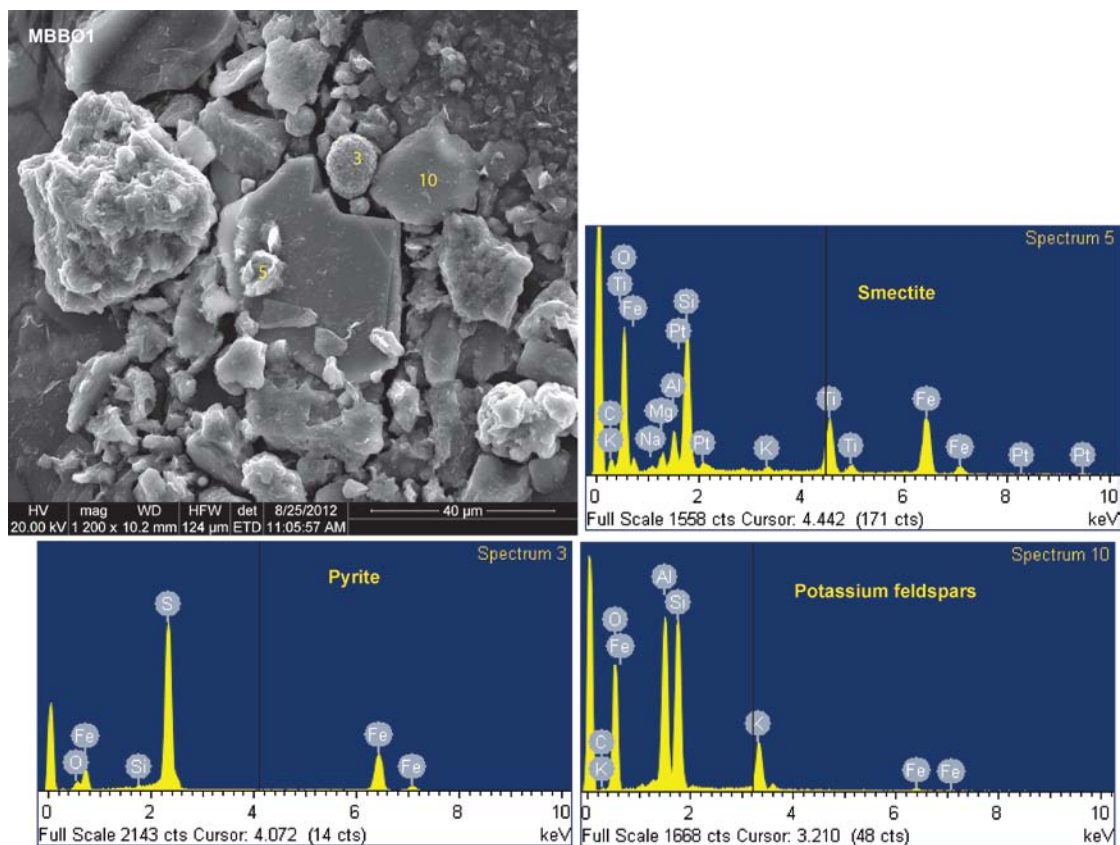
APPENDIX I

SIGNIFICANT (TURKEY'S HSD, $p = <0.05$) NEGATIVE CORRELATION ($r = -0.9914$, & -0.890807) BETWEEN PEPSIN CONTENT AND AFLATOXIN ADSORPTION BY SMECTITES UNDER DIFFERENT pH (TOP), AND SAME pH (BOTTOM) CONDITION.



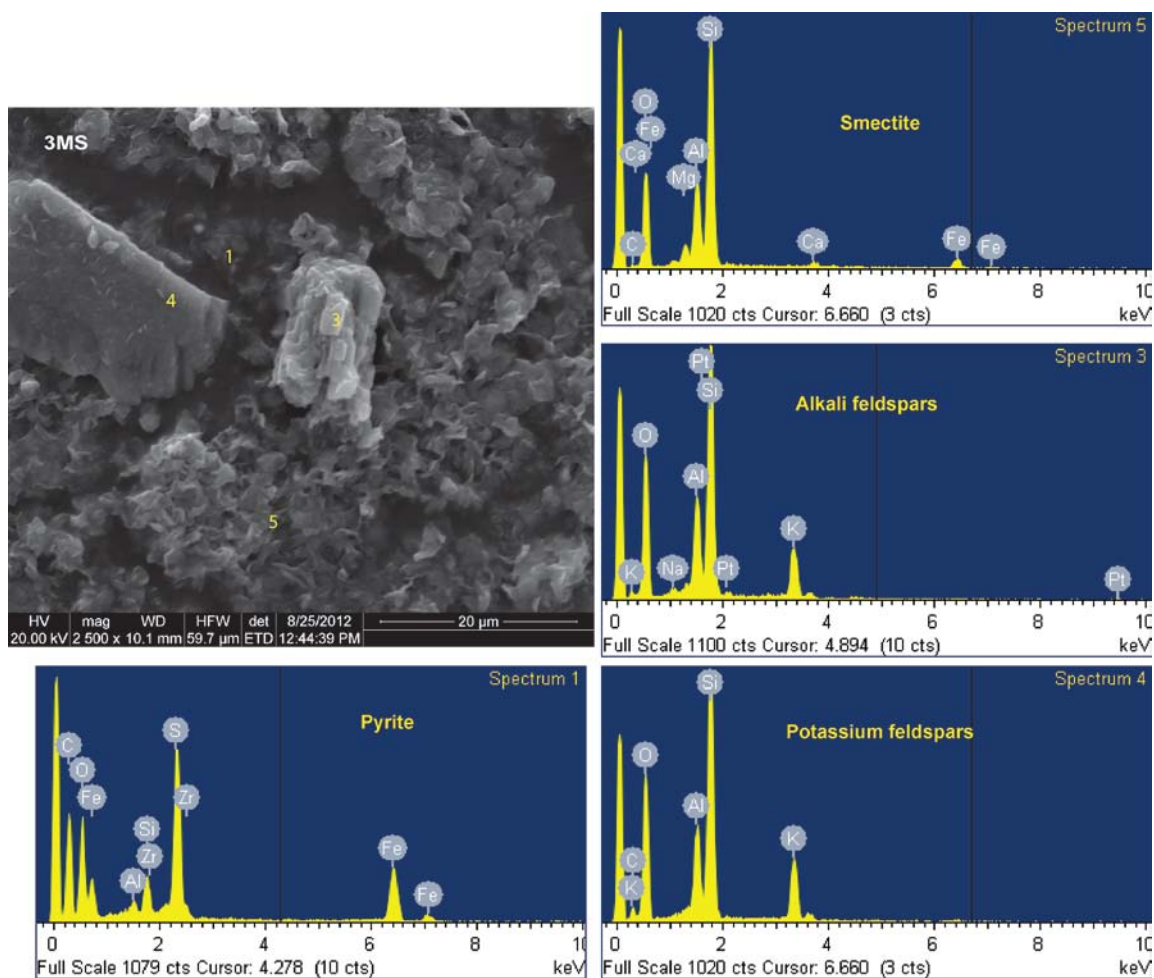
APPENDIX II

SEM/EDS IMAGE (SILT FRACTION) OF AN ALABAMA BENTONITE



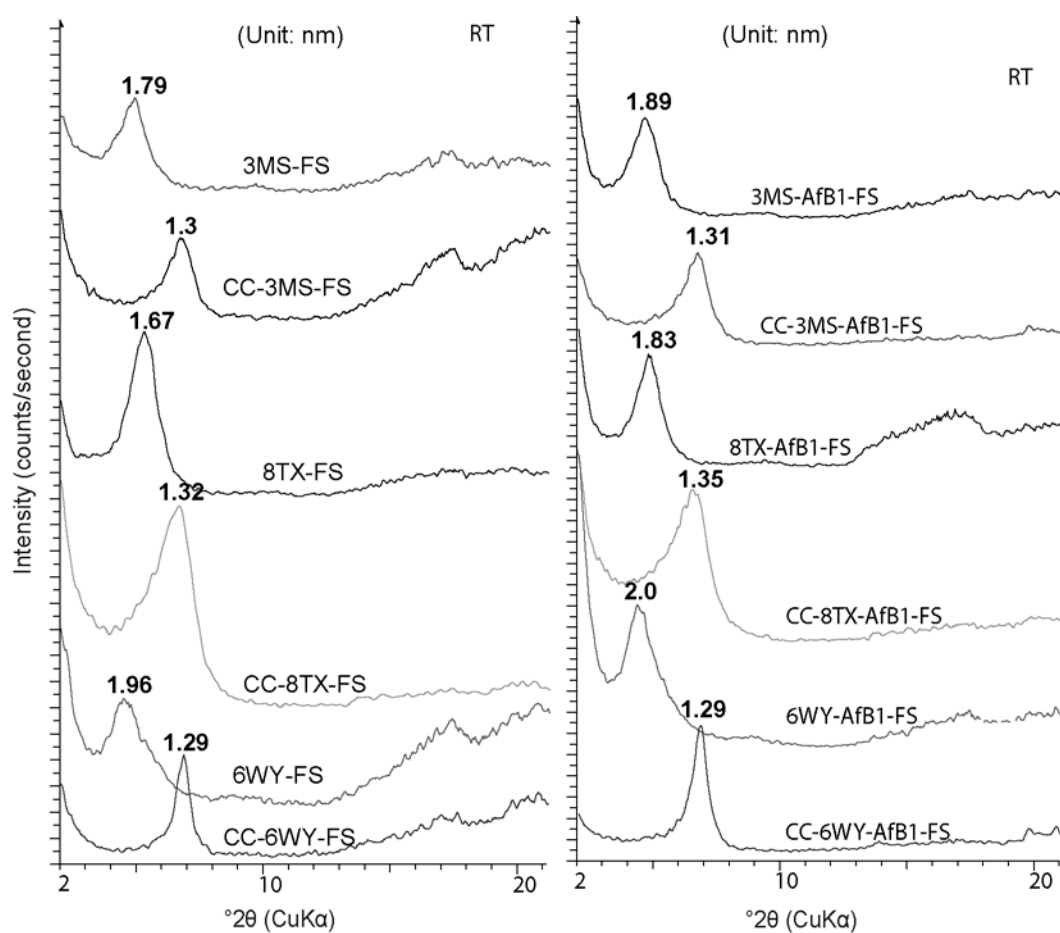
APPENDIX III

SEM/EDS IMAGE (SILT FRACTION) OF A MISSISSIPPI BENTONITE



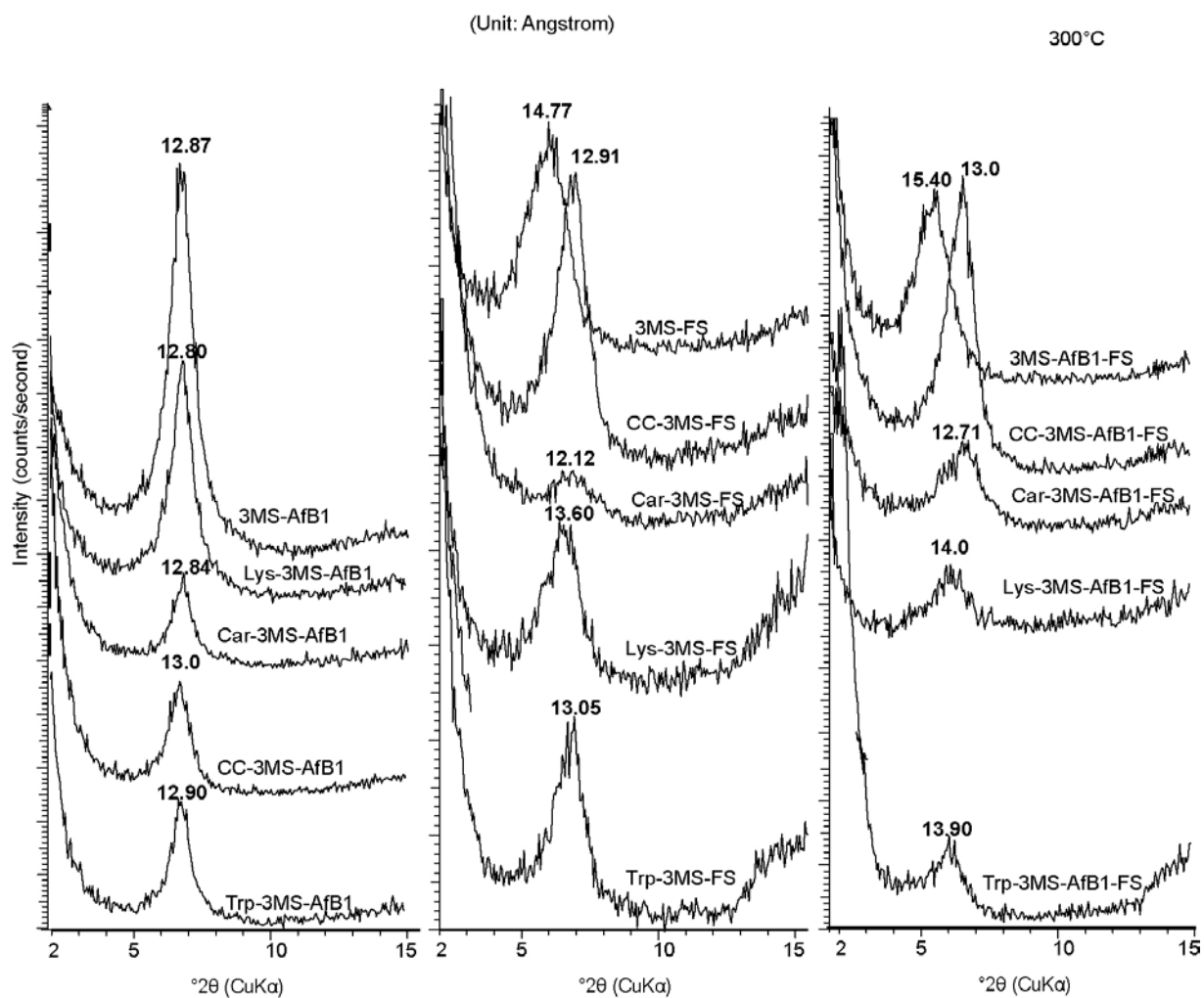
APPENDIX IV

BASAL d_{001} SPACING OF SMECTITE AND ORGANO-SMECTITES AFTER TREATING IN FERMENTATION SOLUTIONS. RECORDED AT ROOM TEMPERATURE



APPENDIX V

BASAL d_{001} SPACING OF SMECTITE AND ORGANO-SMECTITES AFTER TREATING IN DIFFERENT SOLUTIONS. RECORDED AFTER HEATING AT 300 °C



APPENDIX VI

VITA

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| Name: | Sabrina Sharmeen Alam |
| Education: | MS in Soil Science (Thesis). 2009. Department of Soil Science, University of Chittagong, Bangladesh B.Sc. in Soil Science. 2007. Department of Soil Science, University of Chittagong, Bangladesh |
| Professional experiences: | Teaching Assistant: SCSC 301: Soil Science, and SCSC 626: Soil Mineralogy. Department of Soil & Crop Sciences, Texas A&M University. January–December, 2015 Research Assistant: Advisor: Dr. Youjun Deng. Department of Soil & Crop Sciences, Texas A&M University. 2012-2014 Lecturer, Department of Soil Science, University of Chittagong, Bangladesh. 2010-present (on study leave) |
| Research interest: | Use of clay minerals to remediate the environmental contaminants. |
| Articles published and submitted from PhD dissertation: | Sabrina Sharmeen Alam, Youjun Deng, Joe B Dixon. 2015. Minimal interference of glucose and ethanol on aflatoxin B ₁ adsorption by smectites. <i>Applied Clay Science</i> . Vol.104: 143-149. Sabrina Sharmeen Alam, and Youjun Deng. 2016. Aflatoxin B ₁ adsorption by smectites in corn fermentation solution and possible interfering from protein. Submitted to the <i>Applied Clay Science</i> on August, 2016. |
| Awards: | Two research grants and two travel grants from the Clay Minerals Society during 2013-2016. 'Dixon award' 1 st prize for best oral presentation in the student competition at Soil Science Society of America Annual Meeting. 2015. |
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